Beyond chemotherapy

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Beyond chemotherapy: *pro*apoptotic and *anti*angiogenic drug effects in preclinical ovarian cancer models

Thesis, University of Groningen, the Netherlands

ISBN: 978-94-6191-378-4

Cover design and lay-out: A.R.M. van der Bilt
Printed by: Ipskamp Drukkers, Enschede, the Netherlands

The research presented in this thesis was financially supported by the JSM

Printing of this thesis was financially supported by: University of Groningen, University Medical Center Groningen (UMCG), Research Institute for Drug Exploration (GUIDE), Stichting Werkgroep Interne Oncologie

rijksuniversiteit groningen

Beyond chemotherapy: *pro*apoptotic and *anti*angiogenic drug effects in preclinical ovarian cancer models

Proefschrift

ter verkrijging van het doctoraat in de
Medische Wetenschappen
aan de Rijksuniversiteit Groningen
op gezag van de
Rector Magnificus, dr. E. Sterken,
in het openbaar te verdedigen op
woensdag 19 september 2012
om 14.30 uur

door

Arne Roderic Maria van der Bilt

geboren op 27 februari 1986
te Groningen

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CONTENTS

CHAPTER 1

General introduction and Outline of the thesis

GENERAL INTRODUCTION

Currently, several important questions regarding ovarian carcinogenesis and ovarian cancer therapy remain to be answered. While in the past ovarian cancer was thought to arise from the celomic epithelium that lines the surface of the ovaries, molecular profiling studies point towards extraovarian origins.[1] The major histological subtypes distinguished in ovarian cancer (*i.e.* serous, endometrioid, clear cell and mucinous adenocarcinoma) may instead originate from precursor lesions in the Fallopian tube (especially the fimbria) and endometriosis. This contributes to the molecular heterogeneity observed in ovarian cancer, and complicates viewing ovarian cancer as one disease entity in terms of clinical approach.

Ovarian cancer patients commonly present with disseminated disease. Despite current frontline treatment consisting of surgical debulking and chemotherapy, 5-year survival of these patients is still only 25-30%.[2] This can be largely attributed to the development of chemoresistance.

Platinum-based chemotherapy is still the cornerstone of systemic ovarian cancer treatment, and standard chemotherapy – consisting of carboplatin and paclitaxel given in 3-week cycles – has been unchanged since its introduction more than a decade ago. Many alternative regimens have been assessed, but failed to show superior outcome.[3] Some therapeutic improvement might be realized through alternatively dosing current standard chemotherapy, as has been shown for weekly dose-dense paclitaxel administration. However, chemotherapy seems to have reached its ceiling in ovarian cancer treatment.[4] Interest has therefore turned to the potential of incorporating molecular targeted agents that interfere with biological processes that are characteristic for the development and progression of (ovarian) cancer. Two of these hallmark phenomena are induction of angiogenesis (blood vessel formation) and evasion of apoptosis (programmed cell death).[5] Several drugs have been developed to inhibit angiogenesis or induce apoptosis.

Angiogenesis is an important feature of both ovarian physiology and ovarian carcinogenesis. These cancers are considered highly angiogenic, showing extensive vascularization and overexpression of many factors involved in angiogenesis regulation. The importance of angiogenesis in facilitating tumor growth and dissemination has been recognized ever since the early 1970s, when it was also proposed as a potential target for future cancer therapy.[6] It was the discovery of vascular endothelial growth factors (VEGFs) and their receptors (VEGFRs), which are important orchestrators of angiogenesis, that paved the way for antiangiogenic drug

development.[7] Most clinical experience today is based on studies with the VEGF-A-blocking antibody bevacizumab and VEGFR-targeted tyrosine kinase inhibitors (TKIs). These agents demonstrated clinical efficacy in a subgroup of ovarian cancer patients.[8-10] Newer insights have implied numerous other proteins in angiogenesis regulation, providing new avenues for angiogenesis inhibition. The exact place of antiangiogenic drugs in the treatment of ovarian cancer patients is currently being evaluated in a large number of clinical trials.

The tumor suppressor protein p53 mediates chemotherapy-induced cell death via an intrinsic (mitochondrial) mechanism. Inactivating mutations in the *p53 gene* occur frequently in high-grade ovarian cancers, especially in serous adenocarcinoma (up to 95%) which is the most common histological subtype.[11] Mutant p53 status contributes to chemoresistance and patients with mutant p53 cancers have a shorter progression-free and overall survival compared to those with wild-type p53.[12] Apart from intrinsic apoptosis induction, a cancer cell can be triggered to undergo apoptosis by exposure to death ligands, either in an autocrine or paracrine manner. After binding of these ligands to their receptors, which are present on the membrane of many cancer cells including ovarian cancer cells, the extrinsic apoptosis pathway is activated. Members of the tumor necrosis factor (TNF) superfamily are well-known death ligands that are being explored as anticancer drugs, amongst which are TNF-related apoptosis inducing ligand (TRAIL) and Fas ligand (FasL).[13]

The armamentarium of drugs targeting angiogenesis and apoptosis, as well as drugs directed against other hallmarks of cancer biology, is expanding rapidly. Given the heterogeneity in ovarian cancer, it is likely that any molecular targeted agent will only benefit a subgroup of ovarian cancer patients. This notion is supported by results from several clinical trials applying a wide range of molecular targeted agents, all showing – if any – benefit in a subpopulation of ovarian cancer patients. Together with toxicity and cost-effectiveness concerns, this stresses the need for adequate patient selection strategies. Discovering relevant predictive biomarkers would greatly facilitate proper patient selection. This underscores the need for uncovering crucial (cellular) determinants of response to these agents.

This thesis describes preclinical work performed to evaluate the effects of antiangiogenic and proapoptotic death receptor-targeted agents in ovarian cancer models *in vitro* and *in vivo*, and analysis of potential predictive biomarkers in human ovarian cancers.

OUTLINE OF THE THESIS

Amongst the many targeted drugs explored in ovarian cancer, angiogenesis inhibitors have been at the forefront of being integrated in the treatment of ovarian cancer patients. By far the most experience to date stems from studies with drugs blocking VEGF-driven angiogenesis, either by blocking VEGF family ligands using the VEGF-A-blocking antibody bevacizumab or the fusion decoy receptor VEGF-Trap, or by interfering with VEGFR signaling activity using TKIs. In **Chapter 2** we review the available experience from phase II/III clinical trials with these agents. Published studies as well as preliminary reports presented at the American Society of Clinical Oncology (ASCO) meetings and protocols of ongoing trials (extracted from clinicaltrials.gov) are summarized.

Both bevacizumab and VEGFR-targeted TKIs harbor the potential of suppressing VEGF-driven angiogenesis, albeit in distinct ways. Bevacizumab neutralizes VEGF-A, which is considered to be the most prominent proangiogenic ligand within the VEGF family. VEGFR-targeted TKIs have the potential of inhibiting angiogenic signaling relayed by multiple VEGFRs, which can be activated by VEGF-A as well as several other VEGF family members including VEGF-B, VEGF-C and VEGF-D. It is currently unclear how to select patients that are likely to benefit from these treatments, or how to choose between treatment with bevacizumab or a VEGFR-targeted TKI. Insight in the expression of multiple VEGF family members may guide these decisions. In **Chapter 3**, we analyzed the simultaneous protein expression of VEGF-A, VEGF-B, VEGF-C and VEGF-D in a large series of primary ovarian cancers and their omental metastases. To this end, we performed immunohistochemical staining for all four VEGF family members on tissue microarrays (TMAs) containing treatment-naïve ovarian cancer tissues from 270 patients. Staining intensities were analyzed and coupled to a comprehensive clinical database to assess correlations with clinicopathological parameters and patient survival.

Several alternative targeted agents are being explored preclinically for their potential to inhibit ovarian cancer angiogenesis. The mammalian target of rapamycin (mTOR) controls translation of several oncogenic proteins, including several factors involved in angiogenesis.[14] mTOR as well as other components of the mTOR pathway, including the central relay kinase Akt, are often activated in ovarian cancers. Inhibiting mTOR results in translational repression of VEGF-A and its transcriptional regulator hypoxia

inducible factor-1α (HIF-1α). Monitoring reductions in tumor VEGF-A expression, as an early read-out of antitumor efficacy, could potentially provide a means of non-invasive early response prediction which ultimately benefits patient selection. In **Chapter 4**, we monitored tumor VEGF-A levels *in vivo* with positron emission tomography (PET) before and during treatment with the mTOR inhibitor everolimus in a human ovarian cancer xenograft-bearing mouse model. Human ovarian cancer cells were xenografted in mice and tumor VEGF-A levels were measured before and after 2 weeks of continuous everolimus treatment using radiolabeled bevacizumab (^{89}Zr-bevacizumab). Results obtained with ^{89}Zr-bevacizumab PET imaging were correlated to *ex vivo* biodistribution data and tumor VEGF-A protein levels as measured with enzyme-linked immunosorbent assays on whole tumor lysates. Immunohistochemistry was performed to assess vascular density, tumor viability and activation of the downstream mTOR target protein S6.

Bypassing or overcoming chemoresistance is an important objective to improve ovarian cancer prognosis. Targeting tumor cells by activating proapoptotic members of the tumor necrosis factor (TNF) receptor family may provide a potential cellular pathway that can be used to circumvent chemotherapy resistance. Recombinant human TRAIL (rhTRAIL) and TRAIL receptor-targeted antibodies are in clinical trials to analyze their antitumor activity. These agents showed modest activity with limited toxicity in single-agent phase I-II trials as well as in combination studies with chemotherapy.[15] Combining classical chemotherapeutic drugs with rhTRAIL has the potential to act synergistically and overcome resistance to either of the agents alone. Gaining insight in the factors involved in rhTRAIL sensitivity may aid in predicting drug efficacy and finding rational combination therapies.[16] In **Chapter 5** the molecular determinants of rhTRAIL sensitivity and the mechanisms involved in synergy between platinum chemotherapy and rhTRAIL are investigated in an isogenic ovarian cancer cell line model, using cell survival and apoptosis assays, flow cytometry for death receptor expression, analysis of caspase expression and activation with Western blotting and PCR, as well as short interfering RNA (siRNA).

Fas is another member of the TNF receptor family which can be effectively targeted to induce apoptosis in cancer cells. However, *in vivo* systemic use of recombinant human Fas ligand (rhFasL) or anti-Fas antibodies results in marked liver toxicity and these agents are therefore not being tested clinically. A hexameric form of soluble FasL,

called MegaFasL (APO010), has been constructed to decrease liver toxicity, while conserving the capacity to activate the extrinsic apoptosis pathway through binding with Fas.[17] In **Chapter 6** we determined the apoptosis-inducing potential of APO010 in 12 human solid tumor cell lines with various resistance profiles, including platinum sensitive and resistant ovarian cancer cell lines, to assess its ability to circumvent resistance to chemotherapy and other death receptor-targeted drugs. Sensitivity to APO010 was established using cell survival and apoptosis assays, performed on cells treated with single-agent APO010 or APO010 in combination with chemotherapeutic drugs. Fas membrane expression was analyzed with flow cytometry to assess any correlation with APO010 sensitivity. To this end, expression of components of the death-inducing signaling complex (DISC), which is the upstream initiator complex in the extrinsic apoptosis pathway, was also analyzed using Western blotting and PCR analysis.

Finally, a summary of all results presented in this thesis is provided in **Chapter 7**. This is followed by a discussion on the interpretation and (clinical) implication of these findings, along with a discussion on the perspectives for future research.

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CHAPTER 2

TURNING PROMISE INTO PROGRESS FOR ANTIANGIOGENIC AGENTS IN EPITHELIAL OVARIAN CANCER

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Crit Rev Oncol Hematol* 2012 *(In press)

ABSTRACT

Despite efforts to improve chemotherapeutic efficacy in epithelial ovarian cancer, outcome for patients with advanced disease has remained unchanged since the introduction of standard carboplatin and paclitaxel. Interest has therefore shifted toward molecularly targeted therapies that interfere with important features of ovarian carcinogenesis, such as angiogenesis. Several angiogenesis inhibitors, targeting vascular endothelial growth factor (VEGF) ligands (bevacizumab, VEGF-Trap) or their receptors (VEGFR-targeted tyrosine kinase inhibitors) have been clinically evaluated. These agents demonstrated efficacy in phase II clinical trials. Results from phase III trials, in which bevacizumab was added to standard frontline chemotherapy, show a modest effect. Although the initial expectations for angiogenesis inhibitors have been tempered, further research is warranted to define their precise place in the treatment of ovarian cancer. This review summarizes the performed and ongoing studies with regard to angiogenesis inhibitors in ovarian cancer, and the available data on biomarkers for response prediction. Preclinical studies evaluating alternative angiogenesis inhibitors will also be discussed.

INTRODUCTION

Epithelial ovarian cancer is the sixth most common form of cancer in women in developed countries. Patients commonly present with disease that has disseminated beyond the ovaries, due to the absence of symptoms in early-stage disease. Despite tumor response rates of more than 80% to frontline therapy, consisting of surgical debulking and chemotherapy, chemoresistant recurrences are common and result in a poor overall survival (OS) rate of ovarian cancer patients.[1,2]

A carboplatin-paclitaxel doublet is the standard frontline chemotherapy since the past decade. Various chemotherapeutic regimens, including the addition of cytotoxic drugs to standard chemotherapy, failed to show therapeutic superiority.[3,4] Moreover, no effective treatment has been established for platinum refractory ovarian cancer. This has raised interest in targeted drugs that interfere with characteristic phenomena relevant to ovarian cancer biology.

Several antiangiogenic drugs are currently in advanced stages of clinical development. Ovarian cancers are highly angiogenic and belong to the few cancer types in which even monotherapy with antiangiogenic drugs has antitumor activity.[5] Recent phase III results with the vascular endothelial growth factor (VEGF) neutralizing antibody bevacizumab, added to standard frontline chemotherapy, have tempered initial expectations.[6,7] Here, we present the currently available data on the efficacy of angiogenesis inhibitors in ovarian cancer.

ANGIOGENESIS

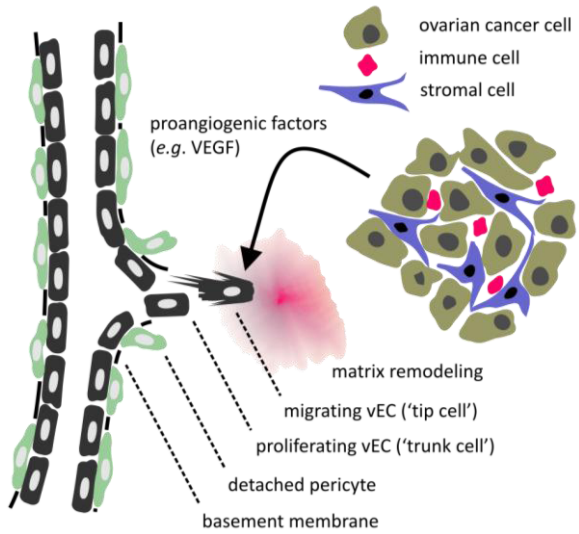
Angiogenesis is the process during which new capillaries arise from pre-existing vessels. In humans, the turnover of vascular endothelial cells (vECs) is low and vessels are quiescent in most healthy adult tissues, whereas angiogenesis is mostly associated with pathological conditions such as cancer.[8] This differential requirement for angiogenesis in health and disease offers a window for antiangiogenic therapy.[8]

ANGIOGENIC SIGNALING

For an extensive description of (cancer) angiogenesis we refer to excellent reviews published on this subject.[8,9] In brief, angiogenesis generally refers to sprouting angiogenesis (**Figure 1**).[10] During this process new vessel sprouts are formed by vECs that make up the endothelial lining of capillaries and post-capillary venules. These vECs loosen their intercellular contacts, proliferate and migrate toward the angiogenic

signal, forming a solid cord of cells. Fragile immature capillaries arise after lumen formation, with vessel walls that only consist of endothelium.[10] Pericytes are recruited to this endothelial lining, establishing direct cellular contacts with the vECs and – together – produce the basement membrane that envelops the matured capillary.

Figure 1. In sprouting angiogenesis, some of the vECs incorporated into established vessels are activated by proangiogenic factors (*e.g.* VEGF-A) produced by both cancer cells and accessory cells (stromal and immune cells). These activated endothelial cells (tip cells) detach from the vascular structure and migrate towards the growth factor gradient. Remodeling of the extracellular matrix through the actions of matrix metalloproteases (MMPs) facilitates their migration. Following the highly motile tip cells are other endothelial cells, called trunk cells, which have the capacity to proliferate and form the new vessel sprout.



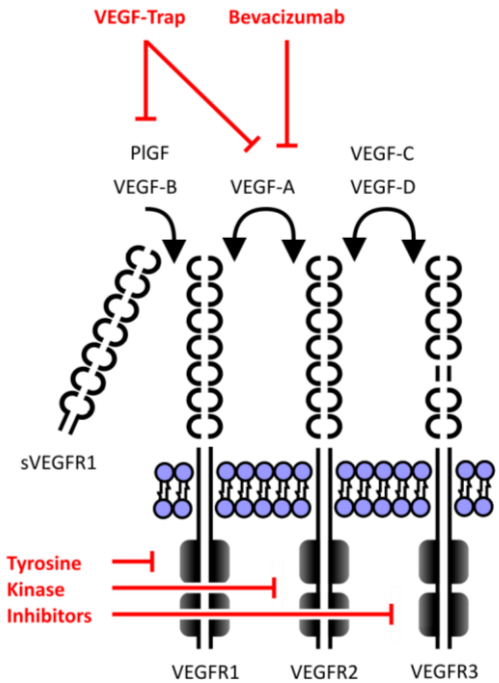


Figure 2. VEGF family members form dimers by covalent, disulfide linkage. These ligand dimers can bind the extracellular parts of VEGFRs, thereby promoting receptor homo- and heterodimerization. VEGF dimers can also interact with several coreceptors, like neuropilins (NRP-1 and NRP-2) and heparan sulphate proteoglycans, enhancing their signaling capacity. The receptor specificity for different VEGF family members is shown schematically. Transphosphorylation of the intracellular kinase domain will occur upon receptor dimerization, followed by recruitment of downstream signal transduction proteins. VEGF(R)-targeted drugs either interfere with ligand-receptor binding or receptor kinase activity (indicated in red and discussed subsequently). Other abbreviations: PlGF, placental growth factor; sVEGFR1, soluble VEGFR1.

Angiogenesis involves the actions of stimulatory and inhibitory cytokines.[9] These cytokines stem from cancer cells as well as stromal and blood cells. VEGF family members are key-drivers of many steps throughout the angiogenic process, from the initial induction of vascular permeability to endothelial cell proliferation, migration and survival.[11] VEGF family members differentially bind to three cognate VEGF receptors (VEGFRs) which are present on the (lymph)vascular endothelium (**Figure 2**). Signaling via VEGFR2 is considered the mainstay of angiogenesis. VEGFR3 contributes to both angiogenesis and lymphangiogenesis.[12] The exact function of VEGFR1 is still subject of debate. At least in part, it serves as a decoy receptor, consistent with its soluble variant sVEGFR1.[13] Taken together, angiogenesis involves signaling by all three VEGFRs and their VEGF ligands.

Quiescent vessels become angiogenic upon alterations in the balance between pro- and antiangiogenic factors, which is called the angiogenic switch.[14] Overexpression of proangiogenic cytokines or downregulation of endogenous angiogenesis inhibitors, such as thrombospondin-1 (TSP-1), will tip the balance in favor of angiogenesis. Hypoxia is a major molecular controller of this switch. Transcriptional regulation of various angiogenesis-related proteins is exerted through hypoxia inducible factors (HIFs) (**Figure 3**).[15,16]

Proangiogenic cytokines produced by cancer cells keep newly formed vessels in a constant angiogenic state. Continuous VEGF production is, in part, driven by the increased metabolic demand for oxygen and nutrients, resulting in HIF transcriptional activity. In addition, oncogenic transformation results in intrinsically induced VEGF production by cancer cells.[17]

Structurally, tumor angiogenesis yields dilated, tortuous and irregularly-shaped blood vessels.[18] The ensuing vasculature is poorly organized, lacking the customary arrangement in arterioles, capillaries and venules. Instead of normal laminar blood flow, the blood flow is chaotic, slow and inefficient.[19] Moreover, these vessel wall abnormalities lead to excessive leakiness and a concomitant increase in interstitial fluid pressure.[20,21] This hampers the exchange of fluid and (macro)molecules between the circulation and the tumor microenvironment.[18] As a consequence, the delivery of therapeutics can be compromised.

ANGIOGENESIS IN OVARIAN CANCER

Angiogenesis plays an important role in ovarian cancer, but is also vital to ovarian physiology.[22] It is imperative for the female reproductive cycle. Studies on primate

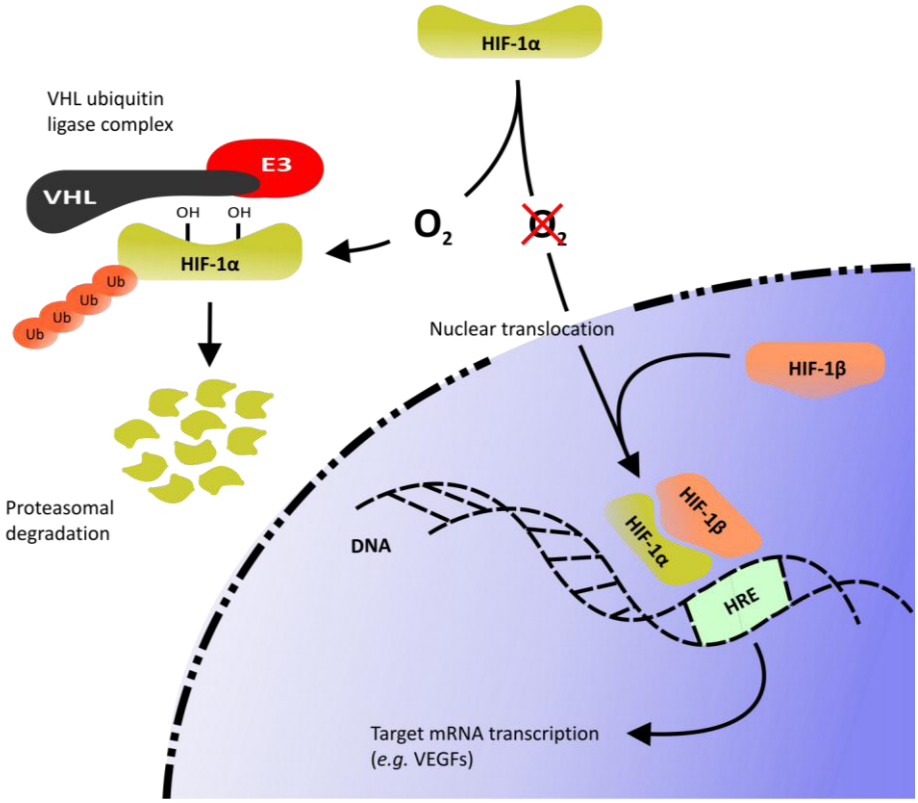


Figure 3. HIFs can form transcriptionally active heterodimers consisting of an oxygen-regulated α-subunit (most commonly HIF-1α) and a constitutively expressed β-subunit (HIF-1β). In the presence of molecular oxygen, HIF-1α is targeted for proteasomal degradation by the association of Von Hippel-Lindau (VHL) ubiquitination complexes to hydroxylated prolyl residues. Under hypoxic conditions, however, stable HIF-1 (*i.e.* the complex formed by HIF-1α and HIF-1β) binds to hypoxia response elements (HREs) within the promoter regions of target genes, a precedent to their transcription. Many genes involved in angiogenesis harbor HREs, including VEGFA. Other abbreviations: E3, E3 ubiquitin ligase; Ub, ubiquitin group.

ovaries propose angiogenesis to underlie the selection of dominant follicles, which are more extensively vascularized than other developing follicles.[23] Similarly, angiogenesis is highly pronounced in the post-ovulatory human corpus luteum.[24] Inhibiting angiogenesis suppresses ovulation and leads to luteal insufficiency.[25,26]

Immunohistochemical staining of ovarian cancer showed that three important human VEGF family members (VEGF-A, VEGF-C and VEGF-D) are widely and often simultaneously expressed in the primary tumor as well as in omental metastases.[27,28] Moreover, VEGF family members are strongly upregulated in ovarian cancers compared to non-malignant disease.[29-31] They are readily detected in ascites of ovarian

cancer patients and are thought to contribute to ascites formation.[32] Besides upregulation of VEGF, HIF-1α protein expression is higher in ovarian cancers compared to benign ovarian disease.[33] HIF-1α expression correlates with VEGF-A expression, suggesting that HIF-1α contributes to VEGF-A overexpression in ovarian cancers.[34] Studies report VEGF-A as well as HIF-1α to be of (independent) prognostic significance in ovarian cancer.[27,30,35]

CLINICAL EXPERIENCE WITH VEGF(R)-TARGETING DRUGS IN OVARIAN CANCER

The pharmacologic possibilities of inhibiting angiogenesis are manifold, but current clinical experience with antiangiogenic drugs in ovarian cancer is limited to the use of VEGF(R)-targeted agents. Clinical trials evaluating the potential of inhibiting either VEGF ligands (bevacizumab, VEGF-Trap) or their receptors (small-molecule tyrosine kinase inhibitors) are discussed below.

BEVACIZUMAB

Bevacizumab, a humanized monoclonal IgG1 antibody which neutralizes VEGF-A, has been most extensively studied. Current experience in ovarian cancer stems from phase II trials in patients with persistent or recurrent disease (**Table 1**) and randomized frontline trials (**Table 2**).

In most studies performed in patients with persistent or recurrent disease, objective response rate was the primary endpoint. In this setting bevacizumab (15 mg/kg intravenously (iv) every 3 weeks until progression or prohibitive toxicity) resulted in single-agent response rates of 16-21%, with median response durations of 4.2 and 10.3 months.[36,37] Even in heavily pretreated, multidrug resistant patients responses to single-agent bevacizumab were objectified.[38]

Antiangiogenic drugs may reduce tumor interstitial fluid pressure and thereby improve chemotherapeutic drug delivery due to normalization of the tumor vasculature.[18,39] Interestingly, bevacizumab efficacy in colorectal cancer patients is only seen when simultaneously administered with chemotherapeutic drugs.[40] The interaction between bevacizumab treatment and chemotherapy delivery in the tumor is currently a topic of interest. Surprisingly, bevacizumab was recently reported to reduce the delivery of radiolabeled docetaxel in patients with lung cancer, thereby possibly reducing chemotherapy efficacy.[41]

Bevacizumab has been given concurrently with chemotherapy in recurrent ovarian cancer patients in several phase II trials (see **Table 1**).[42-46] Although some of

Table 1. Phase II trials evaluating the use of bevacizumab in ovarian cancer.

Ref.	Study design	*N*	Prior lines of chemotherapy	Platinum sensitive *N* (%)	Study outcomes[a]			Common toxicity ≥ grade 3[c] (%)[d]
					RR[b] *N* (%)	PFS (months)	OS (months)	
[36][e]	Single-agent bevacizumab 15 mg/kg iv every 3 weeks	44	2-3, all patients had received topotecan or liposomal doxorubicin	0 (0)	*7/44 (16, all PR)*	4.4 (median)	10.7 (median)	Hypertension (9), proteinuria (16), GI perforation (11), GI obstruction (9)
[37][f]	Single-agent bevacizumab 15 mg/kg iv 3 weeks	62	1-2	26 (42)	*13/62 (21, 2 CR and 11 PR)*	*40% (6-mo PFS)*; 4.7 (median)	16.9 (median)	Hypertension (10)
[38]	Single-agent bevacizumab 10 mg/kg iv every 3 weeks	38	2-9	NR	*9/30*[g] *(30, overall)*	5.9 (median)	8.6 (median)	GI perforation (5), thrombo-embolic event (5)
[42]	Bevacizumab 10 mg/kg iv every 2 weeks and cyclophosphamide 50 mg po daily	70	1-3	42 (60)	17/70 (24, all PR)	*56% (6-mo PFS)*; 7.2 (median)	16.9 (median)	Hypertension (11x), lymphopenia (14x), fatigue (6x), GI obstruction (6x), pain (13x), hyponatremia (5x), vomiting (4x)
[43][h,i]	Bevacizumab 10 mg/kg iv every 2 weeks and topotecan 4 mg/m^2 iv weekly every 3 out of 4 weeks	22	≤ 2	0 (0)	*4/18 (22, all PR)*	NR	NR	Hypertension (17), leucopenia (17)
[44][h]	Bevacizumab 10 mg/kg iv every 2 weeks and nab-paclitaxel 100 mg/m^2 iv weekly every 3 out of 4 weeks	48	≥ 1	0 (0)	18/39 (46, all PR)[j]	8.3 (median)	16.5 (median)	NR[k]
[45]	Bevacizumab 2 mg/kg iv weekly and pegylated liposomal doxorubicin 10 mg/m^2 iv weekly every 3 out of 4 weeks	30	≥ 1	1 (3)	*9/25 (36, 2 CR and 7 PR)*	6.0 (median); 47% (6-mo PFS)	NR	NR[k]
[46][h]	Bevacizumab 10 mg/kg iv every 2 weeks per 4-week cycle, pegylated liposomal doxorubicin 30 mg/m^2 iv and carboplatin AUC 5 iv	54	1	54 (100)	*39/54 (72, NOS)*	14.1 (median)	NR	Hematologic (30, NOS), GI (19, NOS), vascular (15, NOS), hand-foot syndrome (7)

Table 1. *Continued*

Ref.	Study design	*N*	Prior lines of chemotherapy	Platinum sensitive *N* (%)	Study outcomes[a]			Common toxicity ≥ grade 3[c] (%)[d]
					RR[b] *N* (%)	PFS (months)	OS (months)	
[83]	Bevacizumab 15 mg/kg iv every 3 weeks and erlotinib 150 mg po daily	13	≤ 3	7 (54)	*2/13 (15, 1 CR and 1 PR)*	4.1 (median)	11.0 (median)	GI perforation (15), nausea (15), diarrhea (15)
[85]	Bevacizumab 10 mg/kg iv every 2 weeks and erlotinib 150 mg po daily	40	1-7	0 (0)	*9/39 (23, 1 CR and 8 PR)*	4.0 (median); 31% (6-mo PFS)	NR	Skin rash (15), diarrhea (13), fatigue (10), hypertension (8), nasal septal perforation (5)
[124][h]	Bevacizumab 10 mg/kg iv every 2 weeks and temsirolimus 25 mg iv weekly	31	≤ 2	17 (55)	*3/25*[j] *(12, all PR)*	56% (6-mo PFS)	NR	Fatigue (13), stomatitis (13), neutropenia (10), hypertension (6), dehydration (6)
[48]	Carboplatin AUC 5 iv, paclitaxel 175 mg/m^2 iv and bevacizumab 15 mg/kg iv 3 weeks, starting from cycle 2 for 6 cycles	20	None (frontline)	NR	*16/18*[l] *(80, 6 CR and 10 PR)*	NR	NR	Hypertension (10), neutropenia (48)
[49][h]	Oxaliplatin 85 mg/m^2 iv, docetaxel 75 mg/m^2 iv and bevacizumab 15 mg/kg iv every 3 weeks for 6 cycles; followed by 1-yr maintenance bevacizumab	110	None (frontline)	NR	59/95 (62, 31 CR and 28 PR)[j]	*70% (1-yr PFS)*	NR	Neutropenia (39), leucopenia (11), hypertension (9), fatigue (7)
[50]	Carboplatin AUC 5 iv, paclitaxel 175 mg/m^2 iv and bevacizumab 15 mg/kg iv every 3 weeks, starting from cycle 2 for 6-8 cycles; followed by 1-yr maintenance bevacizumab	62[m]	None (frontline)	NR	*47/62*[l] *(76, 13 CR and 34 PR)*	29.8 (median)	NR	Neutropenia (23), thrombopenia (6), metabolic (13, NOS), neuropathy (6), allergic reaction (6), hypertension (10); on maintenance, only hypertension (8) was observed

Abbreviations: N, number of patients; iv, intravenously; po (per os), orally; RR, response rate; CR, complete response(s); PR, partial response(s); PFS, progression-free survival; 6-mo PFS, 6-month PFS; 1-yr PFS, 1-year PFS; OS, overall survival; GI, gastrointestinal; NR, not reported; AUC, area under the curve; NOS, not otherwise specified. [a] Primary efficacy endpoint(s) indicated in italics. [b] Clinical responses determined using RECIST-criteria, unless stated otherwise. [c] Toxicity graded according to the NCI-CTC; only toxicities observed in > 1 patient and ≥ 5% of all patients are reported here. [d] When 'x' is stated, the number of events is reported instead of the percentage of patients in which specific toxicity occurred. [e] Study was terminated prematurely due to high incidence of bowel perforations (11.4%); survival analyses are limited to date of study termination. [f] Initial primary endpoint was 6-month PFS, but the study was redesigned to formally assess the efficacy of bevacizumab based on 6-month PFS or response according to RECIST. [g] Response measured by Gynecologic Cancer Intergroup (GCIG) CA125 criteria. [h] Data reported in abstract form only. [i] Primary endpoints also include PFS and OS, which are not reported preliminarily due to incomplete follow-up. [j] No information is provided on how these responses were measured. [k] No serious toxicity (≥ grade 3) was reported in ≥ 5% of patients. [l] Response rate was determined by combining RECIST and Rustin CA125 criteria. [m] Patients had advanced Müllerian tumors: 45/62 (73%) had ovarian cancer, 10/62 (16%) peritoneal cancer, 4/62 (6%) Fallopian tube cancer, 3/62 (5%) uterine cancer.

these trials showed promising response rates, the true value of combining bevacizumab with chemotherapy in recurrent ovarian cancer has to be established in phase III trials. Two randomized phase III studies are evaluating the efficacy of carboplatin/gemcitabine (OCEANS [NCT00434642]) or carboplatin/paclitaxel (GOG213 [NCT00565851]) with or without bevacizumab in platinum sensitive recurrent ovarian cancer. The OCEANS study randomized 484 patients between placebo *versus* bevacizumab (15 mg/kg iv every 3 weeks) in addition to and maintained after 6 cycles of carboplatin (AUC 4 iv, day 1) *plus* gemcitabine (1000 mg/m^2 iv, day 1 and 8). Preliminary data from this trial show increased median progression-free survival (PFS) in the bevacizumab treated patients compared to placebo (12.4 *versus* 8.4 months, hazard ratio (HR): 0.48, 95% confidence interval (CI): 0.39-0.61, $P < 0.0001$), and a 21.1% increased objective response rate (57.4 *versus* 78.5%, $P < 0.0001$).[47] Data on OS are at present too immature. The value of adding bevacizumab to paclitaxel, topotecan or liposomal doxorubicin is being investigated in platinum resistant recurrent disease (AURELIA [NCT00976911]).

Bevacizumab has also been combined with standard chemotherapy in patients with newly diagnosed ovarian cancer, to evaluate whether it improves long-term outcome.[6,7,48-50] Recently, results became available from two randomized phase III studies – GOG218 and ICON7 – addressing the efficacy and tolerability of frontline bevacizumab (**Table 2**).[6,7,51,52] Patients receiving maintenance bevacizumab demonstrated prolonged PFS, which was the primary endpoint in both studies. In GOG218, median PFS with maintenance bevacizumab was 14.1 months *versus* 10.3 months in the control arm (HR: 0.72, 95% CI: 0.63-0.82, $P < 0.001$), while in ICON7 the benefit was even more modest (19.8 *versus* 17.4 months, HR: 0.87, 95% CI: 0.77-0.99, $P = 0.04$). This limited improvement in PFS does not translate into a prolonged OS, although longer follow-up is required for conclusive analyses. Of note, bevacizumab only improves PFS when continued for 36 (ICON7) to 48 (GOG218) weeks after standard chemotherapy. Based on the PFS gains observed in both studies, bevacizumab recently received European Union approval as part of frontline treatment for patients with advanced ovarian cancer in combination with standard chemotherapy.

Because of different population characteristics and different methods of determining disease progression, GOG218 and ICON7 are hard to compare. CA125 elevation was used in GOG218 (not in ICON7) to define disease progression, which was otherwise radiographically (RECIST guidelines) or clinically determined. When cases

Table 2. Phase III trials evaluating frontline bevacizumab in ovarian cancer.

Ref.	Study design	*N*	Study outcomes[a]		Common toxicity[c] (%)
			PFS (months)	OS[b] (months)	
GOG218 [6][d]	*Arm I* Carboplatin (AUC 6 iv, 6 (3-week) cycles), paclitaxel (175 mg/m^2 iv, 6 cycles), placebo (6 cycles) followed by placebo maintenance (16 cycles)	625	*10.3 (median)*	39.3 (median)	Hypertension (7, ≥ grade 2), pain (42, ≥ grade 2), neutropenia (58, ≥ grade 4), VTE (6, all grades)
	Arm II Carboplatin (AUC 6 iv, 6 cycles), paclitaxel (175 mg/m^2 iv, 6 cycles), bevacizumab (15 mg/kg iv cycle 2-6) followed by placebo maintenance (16 cycles)	625	*11.2 (median); NS*[e]	38.7 (median); NS[e]	Hypertension (17, ≥ grade 2), pain (42, ≥ grade 2), neutropenia (63, ≥ grade 4), VTE (5, all grades)
	Arm III Carboplatin (AUC 6 iv, 6 cycles), paclitaxel (175 mg/m^2 iv, 6 cycles), bevacizumab (15 mg/kg iv cycle 2-6) followed by bevacizumab maintenance (16 cycles)	623	*14.1 (median); P < 0.001*[e]	39.7 (median); NS[e]	Hypertension (23, ≥ grade 2), pain (47, ≥ grade 2), neutropenia (63, ≥ grade 4), VTE (7, all grades)
ICON7 [7][f]	*Arm I* Carboplatin (AUC 5-6 iv, 6 (3-week) cycles), paclitaxel (175 mg/m^2 iv, 6 cycles)	764	*17.4 (median)*	86% (1-yr OS)	Hypertension (2, ≥ grade 2), neutropenia (15, ≥ grade 3)
	Arm II Carboplatin (AUC 5-6 iv, 6 cycles), paclitaxel (175 mg/m^2 iv, 6 cycles), bevacizumab (7.5 mg/kg iv cycle 1/2-6[g]) followed by bevacizumab maintenance (12 cycles)	764	*19.8 (median); P < 0.05*[e]	92% (1-yr OS); NS[e]	Hypertension (18, ≥ grade 2), neutropenia (17, ≥ grade 3)

Abbreviations: N, number of patients; iv, intravenously; PFS, progression-free survival; OS, overall survival; 1-yr OS, 1-year OS; AUC, area under the curve; NS, not statistically significant ($P < 0.05$); VTE, venous thromboembolic event.

[a] Primary efficacy endpoint(s) indicated in italics (PFS in both studies).

[b] Overall survival data are premature, with only 24% (GOG218) and 16% (ICON7) of study participant deceased at the time of analysis.

[c] Toxicity graded according to the NCI-CTC (relevant grades are indicated between brackets); only toxicities observed in > 1 patient and ≥ 5% of all patients are reported here.

[d] In addition to published data: GOG218 data presented at the annual meeting of the American Society of Clinical Oncology (ASCO) 2010.

[e] Relevant study arm compared to control (Arm I).

[f] In addition to published data: ICON7 data presented at the annual meeting of the European Society for Medical Oncology (ESMO) 2010 and the annual meeting of the ASCO 2011.

[g] Bevacizumab was omitted in cycle 1 in patients receiving chemotherapy within 4 weeks of surgery.

with CA125-determined disease progression are censored retrospectively from the GOG218 cohort, median PFS in the maintenance bevacizumab arm is 18.0 months *versus* 12.0 months in the control arm (HR: 0.65, 95% CI: 0.55-0.76, $P < 0.001$).[6,51] GOG218 only included patients with macroscopic residual stage III or any stage IV disease, while ICON7 included all stages (high-risk stage I-IIA and all IIB-IV). Subgroup analysis in ICON7 participants with similar tumor stages as in GOG218 demonstrated a PFS of 16.0 months with maintenance bevacizumab, compared to 10.5 months in the control arm (HR: 0.73, 95% CI: 0.60-0.93, $P = 0.002$).[7,52] Interim survival analyses from ICON7 also demonstrated prolonged OS in this subgroup of patients (36.6 *versus* 28.8 months, HR: 0.64, 95% CI: 0.48-0.85, $P = 0.002$).[7] Although such retrospective analyses are speculative, they might suggest that a subgroup of advanced-stage patients is present in both studies which show a PFS benefit of approximately 6 months.

Other randomized phase III studies are currently investigating the potential of combining intravenously administered bevacizumab with (intraperitoneal or intravenous) cis- or carboplatin *plus* paclitaxel (GOG252 [NCT00951496]), weekly intravenous paclitaxel [NCT01167712] or intravenous oxaliplatin *plus* oral capecitabine [NCT01081262] in frontline ovarian cancer therapy.

Miscellaneous dosing schedules have been used for bevacizumab administration in ovarian cancer clinical trials. In combination studies, bevacizumab was generally administered at 10 mg/kg every other week instead of the widely used single-agent regimen comprising 15 mg/kg every 3 weeks (see **Table 1**). However, given the long serum half-life of bevacizumab ($T_{1/2} \approx$ 17-21 days), it is doubtful that different dosing schedules will have a major impact on treatment outcome. This notion is further strengthened by both frontline phase III trials, which show comparable PFS gains when adjusting for differences in study population and response evaluation, despite different dosing regimens (7.5 mg/kg iv every 3 weeks in ICON7 *versus* 15 mg/kg iv every 3 weeks in GOG218).[6,7]

In general, bevacizumab is well tolerated in ovarian cancer. Hypertension and proteinuria are the most frequently observed bevacizumab-related toxicities, similar to studies in other malignancies.[53] Commonly occurring serious adverse events, grade 3 or higher according to the National Cancer Institute's Common Toxicity Criteria (NCI-CTC), are summarized in **Table 1**. Other, less frequently (< 5%) observed events include wound healing complications, (arterial) thromboembolic events and hemorrhage. Most bevacizumab-related toxicities are well manageable. However, gastro-intestinal (GI) perforations are a point of concern. They occur most frequently in patients with

intensive pretreatment and intestinal disease involvement, in particular when resulting in bowel obstruction.[54,55]

VEGF-TRAP

VEGF-Trap is a fusion protein consisting of two extracellular domains derived from VEGFR1 and VEGFR2 linked to the Fc portion of a human IgG1.[56] It acts as a soluble decoy receptor, preventing receptor activation through high-affinity blocking of VEGF-A, VEGF-B and placental growth factor (PlGF, a third VEGF family member capable of activating VEGFR1). In ovarian cancer mouse models, VEGF-Trap reduced ascites formation, tumor burden and metastases.[57] These effects were potentiated by co-administering taxanes.[58] The combination of VEGF-Trap (6 mg/kg) and docetaxel (75 mg/m^2) given iv every 3 weeks has been explored in a phase II trial in patients with recurrent disease.[59] Data from this trial indicate compelling clinical efficacy of combined therapy, with objective responses observed in 25/46 (54%) evaluable patients, including 11 complete responses (CRs).

Preliminary results from a phase II trial in which 215 patients with recurrent ovarian cancer received single-agent VEGF-Trap (2 or 4 mg/kg, iv every 2 weeks) showed that it was well tolerated, with hypertension (9%) and proteinuria (4%) as most common grade 3 or higher toxicities, analogous to phase I data.[60] In this trial, VEGF-Trap demonstrated only limited antitumor activity (7.3% objective partial responses (PRs) at best).[61] However, VEGF-Trap completely resolved or stabilized ascites in 31 out of 40 patients.[61] Phase II data from a pilot study also demonstrated a prolonged interval between repeat paracentesis upon VEGF-Trap treatment (4 mg/kg iv every 2 weeks) in 10 out of 16 patients requiring frequent paracentesis before study entry.[62] Its potential in counteracting ascites and the need for paracentesis has been further evaluated in a larger, placebo-controlled phase II trial in advanced ovarian cancer patients with symptomatic ascites.[63] Patients were randomly assigned to receive either VEGF-Trap (4 mg/kg iv every 2 weeks, *N* = 29) or placebo (*N* = 26). The mean time to repeat paracentesis was prolonged in patients receiving VEGF-Trap compared to the control group (55.1 *versus* 23.3 days, 95% CI: 10.6-53.1, *P* = 0.002), which was the primary study endpoint. Taken together, these studies indicate potential clinical utility of VEGF-Trap in reducing malignant ascites burden in advanced ovarian cancer patients.

VEGFR-TARGETED TYROSINE KINASE INHIBITORS (TKIs)

TKIs inhibit VEGFR signaling by blocking the activity of one or more of the VEGFRs. Among these, sunitinib (SU11248)[64], pazopanib (GW786034)[65], cediranib (AZD2171)[66,67], sorafenib (BAY43-9006)[68-70] and intedanib (BIBF1120)[71] have been investigated in ovarian cancer phase II trials (**Table 3**). *In vitro*, these compounds display variable activities against VEGFR1, VEGFR2 and VEGFR3, but also target a broad spectrum of other kinases – including other important receptor tyrosine kinases (RTKs) involved in angiogenesis, like platelet-derived growth factor receptors (PDGFRα, PDGFRβ) and fibroblast growth factor receptors (FGFR1-4) (**Table 4**).[72] These three subfamilies are not only functionally, but also structurally (and phylogenetically) interrelated.[73] The TKIs that are currently explored in the treatment of patients with ovarian cancer display activity against both VEGFRs and PDGFRs, but differ in their potential to inhibit FGFRs.

VEGFR-targeted TKIs have limited single-agent activity in patients with recurrent ovarian cancer (see **Table 3**). Overall, objective response rates based on RECIST were 5% at best and only PRs were reported.[64,66,68] For studies in which CA125 response criteria were used, higher response rates were reported.[65,67] Pazopanib given to 36 patients with a biochemical relapse after initial platinum-based chemotherapy resulted in 11 responses according to (modified) Gynecologic Cancer Intergroup (GCIG) CA125 criteria, but no RECIST-based responses were observed.[65] Discrepancies between RECIST-based and CA125-based response rates also apply to patients receiving bevacizumab (see **Table 1**).[38,44]

To date, three studies have defined PFS as the primary endpoint for VEGFR-targeted TKI efficacy (see **Table 3**).[68,71,74] Maintenance treatment with intedanib (250 mg twice daily, for up to 9 months) was evaluated in a randomized, placebo-controlled phase II trial in 83 patients that had responded to their last (at least second-line) chemotherapy.[71] The 36-week PFS rate was 16.3% for intedanib *versus* 5.0% for placebo (HR: 0.65, 95% CI: 0.41-1.02, P = 0.06). No differences were observed in OS between both groups. These data suggest that treatment with a VEGFR-targeted TKI modestly delays disease progression. PFS and OS data from other phase II studies are harder to interpret due to lack of a proper (historical) control population (see **Table 3**).

Little is known about combining VEGFR-targeted TKIs with bevacizumab or conventional chemotherapy.[69,75-78] Such studies have only been performed with sorafenib, where combination with gemcitabine or topotecan did not yield significant activity in patients with recurrent disease, with response and survival rates similar to

single-agent TKI (see **Table 3**).[69,78] Neoadjuvantly given sorafenib combined with carboplatin-paclitaxel resulted in life-threatening toxicity following cytoreductive surgery in 3 out of the first 4 patients enrolled, including an anastomotic leak of the rectosigmoid.[77] Given the major importance of angiogenesis to recover from surgery, antiangiogenic drugs might be less suitable in the neoadjuvant setting. The application of VEGFR-targeted TKIs following initial cytoreductive surgery (including maintenance therapy) is currently explored. The efficacy and safety of combining frontline carboplatin-paclitaxel with sorafenib, followed by maintenance therapy, is currently under investigation in a randomized phase II trial [NCT00390611]. Combining carboplatin-paclitaxel with cediranib is being evaluated in relapsed, platinum sensitive ovarian cancer patients (phase III, ICON6 [NCT00544973]).

In a dose-escalation study of sorafenib combined with bevacizumab, a 42% objective response rate was seen in the subgroup of 19 recurrent ovarian cancer patients.[75,76] However, toxicity was also enhanced and frequently required dose reduction. Intermittent dosing of sorafenib (200 mg twice daily, day 1-5 per week) ameliorated side-effects while retaining clinical activity. There is an ongoing phase II trial analyzing intermittent sorafenib *plus* bevacizumab in recurrent ovarian cancer [NCT00436215]. Preliminary results from this study indicate PRs in 24% of patients.[70]

In addition to inhibiting various angiogenesis-related RTKs, TKIs provoke off-target effects by inhibiting RTKs not involved in angiogenesis as well as a variety of non-RTKs and even non-tyrosine kinases.[72] This may contribute to the enhanced toxicity observed with VEGFR-targeted TKIs compared to bevacizumab. Most commonly observed TKI-related toxicities (all grades) in ovarian cancer phase II trials are fatigue (≤ 89%), gastro-intestinal side effects (≤ 91%, mainly diarrhea but also mucositis, nausea/vomiting and abdominal pain) and hypertension (≤ 83%).[64-68,71] Serious adverse events (≥ grade 3 CTC) are also mostly fatigue, diarrhea and hypertension (see **Table 3**). Some side-effects are more commonly seen with specific TKIs. Dermatologic toxicity, especially hand-foot syndrome (HFS), is a common side effect of sorafenib and sunitinib.[64,68,69] Elevated liver enzymes have been reported most commonly with intedanib.[71]

In a phase II trial evaluating the efficacy of single-agent sunitinib in ovarian cancer, the occurrence of fluid accumulation (ascites or effusion) necessitated adjustment of the initial dose of 50 mg daily (intermittently administered 4 out of 6 week cycles, *N* = 17) to 37.5 mg daily (continuous administration, *N* = 14).[64] Missed doses were frequent on both treatment schedules, mainly due to HFS and mucositis.

Table 3. Phase II trials evaluating the use of VEGFR-targeted TKIs in ovarian cancer.

Ref.	Study design	*N*	Prior lines of chemotherapy	Platinum sensitive *N* (%)	Study outcomes[a]			Common toxicity (≥ grade 3)[c] (%)[d]
					RR[b] *N* (%)	PFS (months)	OS (months)	
[64]	Single-agent sunitinib 50 mg po daily 4 out of 6 weeks, or 37.5 mg po daily continuous due to toxicity	31	1-2	22 (73)	*1/28 (4, all PR)*	4.1 (median)	NR	Lymphopenia (5x), abnormal leukocyte/ granulocyte/platelet counts (10x), fatigue (3x), hand-foot syndrome (3x), diarrhea (3x)
[65]	Single-agent pazopanib 800 mg po daily	36	1-2, patients were required to have relapsed after complete CA125 response to frontline treatment	27 (75)	*11/36*[e] *(31)*	17% (6-mo PFS)	NR	Fatigue (11), diarrhea (8), ALT elevation (8), yGT elevation (11)
[66][f]	Single-agent cediranib 45 mg po daily, reduced to 30 mg po daily due to toxicity[g]	60	≤ 1	26 (43)	*2/41*[h] *(5, all PR)*	4.1 (median)	11.9 (median)	Hypertension (33), fatigue (20)
[67]	Single-agent cediranib 45 mg po daily, reduced to 30 mg po daily due to toxicity[g]	47	≤ 3	16 (35)	*8/47*[i] *(17, all PR)*	5.2 (median); 17% (6-mo PFS)	16.3 (mean); median not yet reached	Hypertension (46), fatigue (24), diarrhea (13), hyponatremia (7)
[68]	Single-agent sorafenib 400 mg po twice daily	73	1-2, patients were required to have progressed within 12 months after completing platinum-based therapy	21 (30)	2/59 (3, all PR)	*24% (6-mo PFS)*; 2.1 (median)	16.3 (median)	Dermatologic (20, of which 10 rash), metabolic (14, NOS), hand-foot syndrome (13)
[74][j]	Single-agent sorafenib 400 mg po twice daily	11	2	4 (36)	0/11 (0)	*2.0 (median)*	11.8 (median)	(None)
[69]	Sorafenib 400 mg po twice daily and gemcitabine 1000 mg/m^2 iv weekly, 3 out of 4 week cycles after first 4 weeks	43	≤ 3, patients must be gemcitabine naïve	1 (2)	*2/43*[k] *(5, all PR)*	5.4 (median)	13.0 (median)	Lymphopenia (28), thrombopenia (19), neutropenia (26), fatigue (16), diarrhea (12), hand-foot syndrome (19), hypertension (7), AST elevation (5)

Table 3. *Continued*

Ref.	Study design	*N*	Prior lines of chemotherapy	Platinum sensitive *N* (%)	Study outcomes[a]			Common toxicity (≥ grade 3)[c] (%)[d]
					RR[b] *N* (%)	PFS (months)	OS (months)	
[70][f]	Sorafenib 200 mg po twice daily, day 1-5/week and bevacizumab 5 mg/kg iv every 2 weeks	30	NR	NR	*6/25 (24, all PR)*	NR	NR	Hypertension (47), thrombosis (13, 3 DVTs and 1 PE), elevated liver enzymes (7)
[78]	Sorafenib 400 mg po daily and topotecan 3.5 mg/m^2 iv weekly, 3 out of 4 week cycles	14	≤ 3	0 (0)	*1/14 (7, 1 PR)*	NR	NR	Neutropenia (38), thrombopenia (15), fatigue (15)
[71][f]	Randomised 2-arm design: patients received maintenance treatment with intedanib 250 mg po twice daily (*n* = 43) *vs* placebo (*n* = 40)	83	2-4, patients must have responded to last chemotherapy	NR	NR	*16.3% vs 5.0% (36-wk PFS)*	NR	Diarrhea (9 *vs* 3), abdominal pain (9 *vs* 8), constipation (7 *vs* 10), ascites (7 *vs* 10), AST elevation (14 *vs* 3), ALT elevation (37 *vs* 0), yGT (44 vs 3), hyponatremia (7 *vs* 5)
[79][f,l]	Single-agent cabozantinib 100 mg po daily for 12 weeks lead-in stage, continued or halted afterwards based on response[m]	70	≤ 3	36 (51)	*17/70*[n] *(24, 1 CR and 16 PR)*	NR	NR	Diarrhea (10), fatigue (9), hand-foot syndrome (7)

Abbreviations: N, number of patients; iv, intravenously; po (per os), orally; RR, response rate; CR, complete response(s); PR, partial response(s); PFS, progression-free survival; 6-mo PFS, 6-month PFS; 36-wk PFS, 36-week PFS; OS, overall survival; GI, gastrointestinal; NR, not reported; NOS, not otherwise specified; AST, aspartate transaminase; ALT, alanine transaminase; yGT, gamma glutamyl transpeptidase; DVT, deep venous thrombosis; PE, pulmonary embolism. [a] Primary efficacy endpoint(s) indicated in italics. [b] Clinical responses determined using RECIST-criteria, unless stated otherwise. [c] Toxicity graded according to the NCI-CTC; only toxicities observed in > 1 patient and ≥ 5% of all patients are reported here. [d] When 'x' is stated, the number of events is reported instead of the percentage of patients in which specific toxicity occurred. [e] Responses based on modified GCIG CA125 criteria; in 17 pts with radiographically measurable disease no responses following RECIST criteria were noted. [f] Data reported in abstract form only. [g] Dose reductions to 30 mg po daily [66,67] and 20 mg po daily [66] were required due to toxicity. [h] Two additional unconfirmed responses have also been reported. [i] Responses were measured either by RECIST-criteria or by modified GCIG CA125 criteria. [j] Trial was stopped after first stage of accrual due to inefficacy of the study drug. [k] GCIG CA125 responses were observed in 28% of patients. [l] Data from the accompanying oral presentation were incorporated into the table. [m] Patients with a CR or PR continued on open-label extension, patients with stable disease were randomised between cabozantinib versus placebo, while patients with disease progression discontinued. [n] Response rate as determined after the 12-week lead-in stage.

Responses (1 PR according to RECIST criteria and 3 CA125-based responses) were only observed at the higher dose level. A similar scenario was seen with single-agent cediranib, with toxicity (especially fatigue and diarrhea) requiring reduction of the starting dose (45 mg daily) to 10-30 mg daily in clinical trials.[66,67] In ovarian cancer patients, pazopanib 800 mg daily was also poorly tolerated and frequently required dose reduction, dose interruption or drug discontinuation, mainly due to fatigue and elevated liver enzymes.[65] In combination studies of sorafenib *plus* bevacizumab, the maximum tolerated dose of both drugs was much lower compared to recommended, tolerable single-agent doses.[75,76] Toxicity-related dose reductions may hamper clinical efficacy.

Preliminary data show that cabozantinib, a highly potent TKI directed against VEGFR2 and the hepatocyte growth factor (HGF) receptor c-Met, has promising single-agent activity in recurrent ovarian cancer with a reported 24% objective response rate (see **Table 3**).[79] Whether this effect can be attributed solely to angiogenesis inhibition, or originates also from different antitumor effects which occur upon c-Met inhibition, has to be elucidated.[80]

Taken together, the currently available clinical experience with bevacizumab and VEGFR-targeted TKIs indicates modest activity of these agents in ovarian cancer. Discovering predictive biomarkers might facilitate administering these drugs to the subgroup of patients that are likely to respond.[81]

Table 4. Inhibition profiles of different VEGFR-targeted TKIs for VEGFRs, PDGFRs and FGFRs.

	sorafenib [BAY43-9006] IC_{50} **(μM)**[a] [134,135]	**sunitinib [SU11248]** IC_{50} **(μM)**[a] [134]	**cediranib [AZD2171]** IC_{50} **(μM)**[a] [136]	**pazopanib [GW786034]** IC_{50} **(μM)**[a] [134]	**intedanib [BIBF1120]** IC_{50} **(μM)**[a] [137]
VEGFR1/Flt-1	0.009	0.021	0.005	0.007	0.034
VEGFR2/KDR	0.028	0.034	< 0.001	0.015	0.021
VEGFR3/Flt-4	0.007	0.003	≤ 0.003	0.002	0.013
PDGFRα	0.004[b]-0.933	0.143	0.036	0.073	0.059
PDGFRβ	0.010[b]-1.129	0.075	0.005	0.215	0.065
FGFR1	0.064-0.580	0.437	0.026	0.080	0.069
FGFR2	0.825	0.852	NR	0.350	0.037
FGFR3	1.019	0.314	NR	0.138	0.108
FGFR4	NR	NR	NR	NR	0.610

Abbreviations: VEGFR, vascular endothelial growth factor receptor; PDGFR, platelet-derived growth factor receptor; FGF, fibroblast growth factor receptor; NR, not reported; IC_{50}, drug concentration needed to inhibit 50% of kinase activity.

[a] Non-cellular IC_{50} values determined from different biochemical assays (including time resolved fluorescence energy transfer assays).

[b] IC_{50} values were calculated based on K_i values using the Cheng-Prusoff equation, with ATP concentrations set at the *K*m for each kinase.

SELECTING PATIENTS FOR ANTIANGIOGENIC THERAPY

At present, no conclusive data is available on biomarkers that predict response to VEGF(R)-targeted drugs in ovarian cancer.[82] Data from a few phase II bevacizumab studies are available which included exploratory biomarker analyses to unveil determinants of response, but often only in a subgroup of patients.[42,83-85] Consequently, these analyses are underpowered. VEGF-A, the target of bevacizumab, was mostly analyzed in these studies, both in blood samples and tumor tissue. Although bevacizumab often reduces circulating serum VEGF-A levels, this decrement does not predict tumor response.[38,84] Controversy exists with regard to the predictive value of pretreatment circulating (serum and plasma) VEGF-A levels.[38,42,83] In a study of only 30 patients with multiresistant recurrent ovarian cancer, low pretreatment serum VEGF-A predicted better response and longer PFS and OS upon treatment with single-agent bevacizumab.[38] However, this might rather reflect prognostic than predictive value of serum VEGF-A, since lower pretreatment plasma VEGF-A also predicted favorable PFS and OS in several randomized phase III trials in other solid cancers irrespective of adding bevacizumab to chemotherapy.[86] Plasma and serum levels do not reliably reflect the true amount of circulating VEGF-A, since these do not take into account the contribution of platelets which actively sequester substantial amounts of angiogenesis regulators including VEGF-A.[87]

Exploratory biomarker analyses incorporated into the phase II study executed by the Gynecologic Oncology Group (GOG) demonstrated that neither plasma, serum nor tumor cell expression of VEGF-A was predictive for response to bevacizumab in 61 patients with persistent or recurrent ovarian cancer.[84] Tumor biopsies were performed before and during bevacizumab therapy, followed by immunostaining for CD31, VEGF, p53 and TSP1. Low pretreatment CD31-based microvessel density (MVD), a measure for vascularity, was associated with response to bevacizumab.

Angiogenesis-related gene polymorphisms revealed no predictive value. VEGF-A polymorphisms do not relate to bevacizumab efficacy.[38,88] In ovarian cancer patients treated with bevacizumab and metronomic cyclophosphamide, one germline polymorphism emerged as a molecular predictor of response. An interleukin-8 polymorphism (IL-8 A-251T), linked to increased IL-8 production, correlated with lack of response, possibly resulting from IL-8-driven VEGF-independent angiogenesis.[88,89] To date, polymorphism analyses have been performed solely on germline DNA isolated from blood cells and not on tumor material.

RNA microarray analysis of 199 serous ovarian cancers has revealed the existence of 3 ovarian cancer subgroups characterized by distinct gene expression profiles.[90] The differences in gene expression profiles between these subgroups are predominantly attributable to differential expression of angiogenesis-related genes. It is of interest to analyze whether differences in gene expression profiles exist between patients that do or do not benefit from antiangiogenic therapy.

Current knowledge about biomarkers for antiangiogenic treatment largely stems from studies performed on pretreatment tumor tissue samples. These may fail to predict treatment outcome, since changes in biomarker expression during antiangiogenic therapy might be more relevant to therapeutic efficacy. Positron emission tomography (PET) visualizing VEGF-A with radiolabeled bevacizumab or its Fab-fragment ranibizumab (VEGF-PET) enables real-time monitoring of tumor VEGF-A levels.[91,92] In ovarian cancer xenograft models, VEGF-PET visualized changes in tumor VEGF-A levels upon exposure to several angiogenesis inhibitors, including sunitinib.[92,93] Its potential as a non-invasive biomarker providing insight in tumor VEGF-A levels during the course of treatment is currently under clinical investigation.

ALTERNATIVE APPROACHES TO ANTIANGIOGENIC THERAPY IN OVARIAN CANCER

TARGETING ALTERNATIVE PROANGIOGENIC FACTORS

Since the discovery of VEGF-A, several alternative proangiogenic factors have been discovered. These not only include other VEGF family members, but also several unrelated proangiogenic factors relevant to ovarian cancer angiogenesis: FGFs, PDGFs, angiopoietins (Ang-1, Ang-2), interleukins (IL-6, IL-8) and many more. These factors provide potential targets for antiangiogenic therapy.

IL-8 is a potential target for angiogenesis inhibition in ovarian cancer, which is not yet evaluated in clinical trials. Secreted IL-8 can activate endothelial receptors CXCR1/2, which can drive angiogenesis *in vivo* despite the presence of bevacizumab.[94] Inhibition of CXCR1/2 or protease-activated receptor-1 (PAR-1, which regulates IL-8 secretion) by cell-penetrating pepducins (lipidated peptides providing intracellular inhibition of G protein-coupled receptors) inhibits angiogenesis in ovarian cancer xenograft models, which has also been observed upon IL-8 gene silencing using liposome-encapsulated siRNA.[94-96]

TARGETING NON-TUMOR CELLS INVOLVED IN OVARIAN CANCER ANGIOGENESIS

Stromal and bone-marrow derived cells (BMDCs) also contribute to tumor angiogenesis.[97] The precise interplay between these microenvironmental components and tumor cells is yet to be unraveled.

Stromal and bone-marrow derived cells can be incorporated into the vasculature (as progenitor cells) or stimulate angiogenesis by secreting proangiogenic factors.[98] They confer resistance to VEGF-A-neutralizing therapy. This is at least in part due to production of proangiogenic Bv8, a homolog of endocrine gland-derived VEGF, which is structurally unrelated to the VEGF family but has similar proangiogenic effects mediated by G protein-coupled receptors.[99,100] Endocrine gland-derived VEGF is expressed in ovarian cancer, and is derived from non-epithelial components of the tumor including stromal and bone-marrow derived cells.[101]

Among the various cell types that together form the stroma, tumor-associated fibroblasts, which are activated fibroblasts, have received particular attention. Tumor-associated fibroblasts are abundantly present in ovarian cancers and are associated with an increased (lymph)vascular density.[102] Tumor-associated fibroblasts are also extensively recruited into xenografted ovarian cancers, specifically to angiogenic areas.[103] They produce VEGF-A, but also facilitate VEGF-A-independent angiogenesis.[104]

Various cell types involved in angiogenesis, including endothelial (precursor) cells, pericytes and fibroblasts, express PDGF receptors (PDGFRα and PDGFRβ). PDGF-A, which can be produced by ovarian cancer cells, is crucial for PDGFRα-driven tumor-associated fibroblast chemotaxis.[105] Targeting PDGFRα could reduce the formation of angiogenic stroma.

PDGFRβ activation by PDGF-B, which is produced by vECs, recruits pericytes to the vascular endothelium.[106] Pericytes stabilize nascent vessels and regulate endothelial survival. PDGFRβ blockade inhibits pericyte recruitment in the murine corpus luteum as well as in orthotopic ovarian cancer xenografts.[107] However, solely inhibiting PDGFRβ has little (pre)clinical activity. Single-agent imatinib, a PDGFR-targeting TKI, demonstrated minimal activity (1.8% response rate) in a phase II trial performed in patients with recurrent ovarian cancer.[108] Animal studies demonstrated that PDGFB/PDGFRβ inhibition using PDGF-Trap or imatinib does not decrease vascularization, increase endothelial cell apoptosis or reduce tumor growth.[106,109] Instead, disrupting pericyte coverage by PDGFRβ inhibition results in a microvasculature that is particularly dependent on VEGF-mediated survival signals. By

abolishing this survival signal, endothelial apoptosis and vascular regression may follow. In an intraperitoneal HeyA8 ovarian cancer mouse model, combinatorial treatment with VEGF-Trap *plus* PDGF-Trap did result in reduced vascularization, increased endothelial cell apoptosis and reduced tumor burden compared to either treatment alone or control therapy.[106]

TARGETING HIF-1A

By activating HIF-1, hypoxia is involved in many steps of the angiogenic process (see **Figure 3**). HIF-1 target genes include several proangiogenic factors (*e.g.* VEGF-A, PlGF, FGF-2, Ang-2) and their receptors, matrix remodeling factors (*e.g.* MMPs), factors mediating the migration/invasion of bone-marrow derived cells (*e.g.* SDF-1, CXCR4) and regulators of vascular maturation (*e.g.* PDGFs, Ang-1).[110] HIF-1 activation provides cancer cells with mechanisms by which they can adapt to or escape from the detrimental effects of radiotherapy, chemotherapy and angiogenesis inhibition.[97] VEGF(R)-targeted therapies can rapidly decrease tumor blood flow and consequently increase hypoxia, thereby provoking evasive angiogenesis.[111] Such 'escape' angiogenesis might explain the lack of translation of PFS into OS gains in several phase III bevacizumab trials in solid cancers.[112] This might result from the upregulation of proangiogenic factors upon VEGF(R) inhibition, especially with VEGFR-targeted TKIs.[113] It can also be due to enhanced invasiveness and metastatic potential that was observed in several animal models after sunitinib treatment, a cause of great concern.[114,115] Hypoxia is speculated to underlie this treatment-induced phenotypic transformation.[116]

HIF-1 accumulation in ovarian cancer is primarily regulated at the post-transcriptional level, demonstrated by the lack of correlation between protein and mRNA expression of the regulatory subunit HIF-1α.[117] Drugs that interfere with HIF-1α translation or promote HIF-1α degradation, therefore, harbor potential as antiangiogenic strategies (**Figure 4**).

The mammalian target of rapamycin (mTOR) regulates the translation of a multitude of proteins, involved in key cellular processes like proliferation, migration and angiogenesis.[118] Activation of mTOR is common in many cancers, including ovarian cancer.[119] It facilitates translation of specific mRNAs that harbor regulatory elements in their 5'-untranslated regions (UTRs), including HIF-1α and proangiogenic factors like VEGF-A or FGF-2.[120] Inhibitors of mTOR have antiangiogenic properties.[121] Expression of HIF-1α and VEGF-A in ovarian cancer cell lines as well as vascularization *in vivo* are

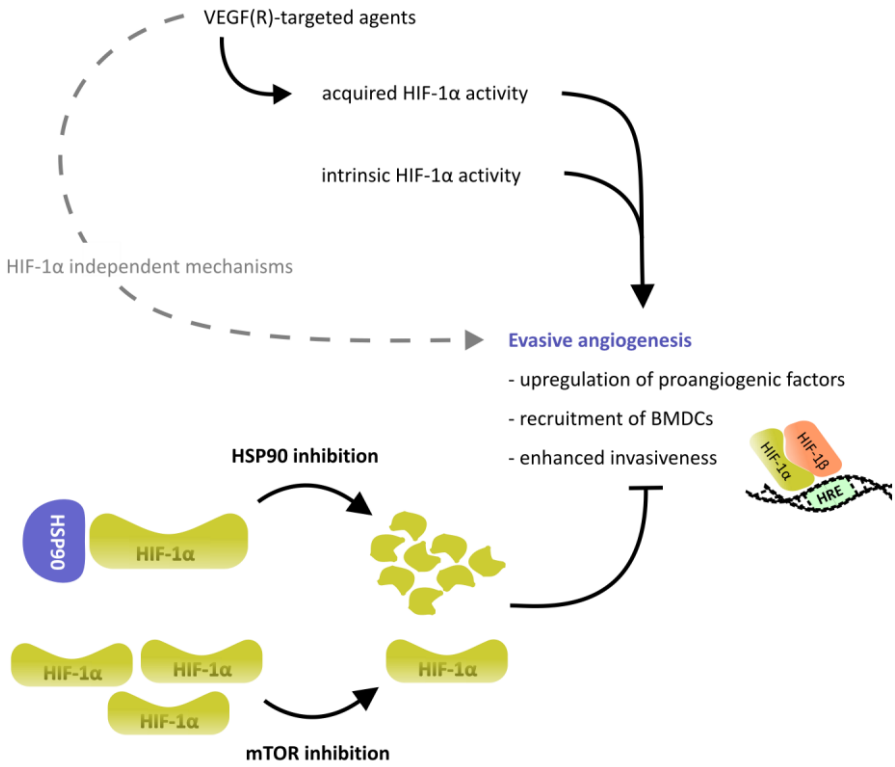


Figure 4. HIF-1 has been linked to drug resistance upon treatment with VEGF(R)-targeted drugs. HIF-1 activity can be intrinsic or acquired in reaction to hypoxia resulting from antiangiogenic therapy. HIF-1 aids ovarian cancer cells in overcoming angiogenesis inhibition by upregulation of multiple proangiogenic factors. Furthermore, HIF-1 activity can enhance the invasive and metastatic potential of these cells by regulating several genes involved in epithelial-to-mesenchymal transition (EMT). Therapeutic strategies that are being explored in ovarian cancer aim at interfering with HIF-1α translation (*e.g.* mTOR inhibitors) or promoting its degradation (*e.g.* HSP90 inhibitors).

reduced upon treatment with the mTOR inhibitor everolimus.[122,123] Preliminary phase II data from a study in which temsirolimus was combined with bevacizumab in the treatment of recurrent ovarian cancer show 56% 6-month PFS and a 12% objective response rate (see **Table 1**).[124] HIF-1α is a crucial determinant for sensitivity to mTOR inhibition in renal cell cancer, but this remains to be established clinically for ovarian cancer.[125] Inhibition of mTOR can also evoke a HIF-1α independent antiangiogenic response through reducing endothelial cell proliferation.[126]

Heat shock protein 90 (HSP90) is a molecular chaperone involved in (conformational) stabilization of a variety of proteins involved in angiogenesis.[127] It is ubiquitously expressed in ovarian cancers.[128] HSP90 associates with cytoplasmic

localized HIF-1α and is essential for hypoxia-induced HIF-1α activity.[129] Various HSP90 inhibitors exhibited antiangiogenic properties in preclinical ovarian cancer studies. Treatment with the HSP90 inhibitor NVP-AUY922 inhibits tumor growth, reduces vascularization and downregulates VEGF-A and HIF-1α expression in ovarian cancer xenografts.[93,130] Irrespective of its impact on proangiogenic factors produced by cancer cells, HSP90 inhibition reduces proliferation, migration, tubular differentiation and VEGFR expression of (human umbilical vein) endothelial cells.[130,131]

Inhibiting mTOR or HSP90 evokes a multifaceted anticancer response including antiangiogenic, antimetastatic, antiproliferative and proapoptotic effects. Ongoing clinical studies will determine their role in the clinic. More selective ways of HIF-1α inhibition pose an interesting avenue for antiangiogenic drug development.

CONCLUSIONS AND PERSPECTIVES

Angiogenesis is an important contributor to ovarian carcinogenesis and progression, providing a clear rationale for the use of antiangiogenic drugs. Yet, clinical data with VEGF(R)-targeted agents that are currently available have not lived up to the initial expectations. Given the substantial costs involved, population-wide use of these drugs is unlikely to be justifiable in terms of generally accepted standards of cost-effectiveness.[132] It is conceivable that clinical implementation is greatly aided by uncovering predictive biomarkers that facilitate administering these expensive drugs to a subgroup of patients that are likely to respond.[81] Possibly, a more comprehensive view on angiogenesis can help to determine which patients might benefit from antiangiogenic therapy. The contribution of different angiogenic factors and cell types involved in ovarian cancer-specific angiogenesis, as well as the mechanisms of resistance to antiangiogenic drugs, might help to select the appropriate targets, and thereby drugs, for individual patients. It is conceivable that therapeutic regimens targeting multiple, distinct aspects of angiogenesis are superior to singular antiangiogenic therapies.[133]

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CHAPTER 3

MULTIPLE VEGF FAMILY MEMBERS ARE SIMULTANEOUSLY EXPRESSED IN OVARIAN CANCER: A PROPOSED MODEL FOR BEVACIZUMAB RESISTANCE

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Curr Pharm Des* 2012 *(In press)

ABSTRACT

BACKGROUND Insight into the expression of multiple vascular endothelial growth factor (VEGF) family members can support the implementation of antiangiogenic therapy. This study aimed to assess VEGF family member expression in ovarian cancers and related omental metastases.

METHODS Tissue microarrays encompassing 270 primary cancers and 112 paired metastases were immunostained for VEGF-A, VEGF-B, VEGF-C and VEGF-D. Staining intensities were categorized as absent, weak, moderate or strong. Expression was related to clinicopathological characteristics and survival.

RESULTS Immunohistochemical positivity (defined as moderate or strong expression) was observed for VEGF-A in 90%, VEGF-B in 4%, VEGF-C in 41% and VEGF-D in 55% of the primary ovarian cancers. VEGF-A expression correlated with VEGF-C and VEGF-D expression ($P < 0.01$). Simultaneous positivity for VEGF-A and VEGF-C or VEGF-D was observed in 38% and 54% of the cancers, respectively. Metastases showed positivity for VEGF-A in 78%, VEGF-B in 5%, VEGF-C in 26% and VEGF-D in 45% of cases. VEGF family member expression showed no independent prognostic significance in multivariate survival analysis.

CONCLUSION VEGF-A, VEGF-C and VEGF-D are widely and often simultaneously expressed in ovarian cancer, which may contribute to bevacizumab resistance. Measuring their expression could support a rational, individualized choice of antiangiogenic therapy and might be of predictive value. Studies are warranted to determine whether combinatorial analysis of VEGF family member expression can be used to predict antiangiogenic drug efficacy.

INTRODUCTION

Ovarian cancer has the highest mortality amongst the gynecologic malignancies.[1] Many chemotherapeutic regimens have been explored since the introduction of carboplatin and paclitaxel as frontline chemotherapy after surgical debulking, but none has been shown to further improve clinical outcome. Patients presenting with advanced-stage disease, which account for almost 80% of all ovarian cancer patients, still face a disappointing 30% 5-year survival rate. To improve their prognosis, alternative treatment strategies are clearly needed.

Targeting tumor angiogenesis constitutes an interesting new approach in the treatment of ovarian cancer. Ovarian cancers are considered to be highly angiogenic, depending on the establishment of a functional vasculature that enables them to grow invasively and to metastasize. To ensure adequate perfusion, cancer cells produce proangiogenic vascular endothelial growth factors (VEGFs). Four human VEGFs are distinguished within the VEGF family – VEGF-A, VEGF-B, VEGF-C and VEGF-D – which can bind to three VEGF receptors (VEGFRs) present on the vascular endothelium.[2]

VEGF-A-mediated VEGFR2 activation has long been considered the predominant mode of angiogenic signal transduction, which led to the development of the monoclonal anti-VEGF-A antibody bevacizumab. Response rates up to 21% have been reported in ovarian cancer patients receiving single-agent bevacizumab for disease recurrences.[3,4] Preliminary phase III data show that addition of bevacizumab to frontline carboplatin and paclitaxel followed by maintenance bevacizumab prolongs progression-free survival (PFS) in patients with advanced ovarian cancer.[5,6] Refined insights have implied all VEGFRs in angiogenesis, shifting interest also to other VEGF family members and fueling the development of small-molecule tyrosine kinase inhibitors (TKIs) to target multiple, different VEGFRs.[2,7,8] Several VEGFR-targeted TKIs have shown single-agent efficacy in recurrent ovarian cancer and have entered phase III evaluation.

Only a subgroup of patients will benefit from antiangiogenic therapy. Angiogenesis inhibitors are not sufficiently (cost-)effective to merit their general use in ovarian cancer and clinical implementation will depend on the discovery of predictive biomarkers. The selection of patients who are likely to respond to antiangiogenic therapy – as well as choosing which antiangiogenic drug is most suitable – is aided by a better understanding of the role played by the different VEGF family members. Previous studies addressing the expression of VEGF family members in ovarian cancer

have mostly focused on VEGF-A, leaving the expression of other VEGF family members subject of debate.

In this retrospective study we determined the expression of VEGF-A, VEGF-B, VEGF-C and VEGF-D in a large, well-defined cohort of ovarian cancer patients, using tissue microarrays (TMAs) comprising tissue from primary ovarian cancers and their omental metastases. These expression data were coupled to a comprehensive clinical database to assess correlations with common clinicopathological characteristics as well as to investigate the prognostic value of the different VEGF family members.

METHODS

STUDY POPULATION AND TISSUE ACQUISITION

Between March 1982 and June 2006 tumor tissue samples were collected from patients diagnosed with primary epithelial ovarian cancer. All patients primarily underwent cytoreductive surgery, during which they were staged according to International Federation of Gynecology and Obstetrics (FIGO) criteria. If indicated, chemotherapy was prescribed according to clinical guidelines. Informed consent was obtained from all patients for data storage and tumor collection, and studies were conducted in accordance with the Declaration of Helsinki principles and institutional review board policies.[9]

Surgically resected tissue was assessed by a gynecological pathologist. Primary ovarian cancers were classified according to histological subtype and graded based on World Health Organization (WHO) criteria. Tissue samples were fixated in formalin overnight and subsequently embedded in paraffin blocks.

A comprehensive, anonymous database has been kept for this patient cohort containing information on several clinicopathological variables. Notably, this database includes information on disease stage, tumor differentiation grade, tumor histology, treatment parameters and patient survival. Prior to our analyses, the database was updated with follow-up registry.

TISSUE MICROARRAY CONSTRUCTION

TMAs were constructed as described previously.[9] In summary, four needle core biopsies (Ø 0.6 mm) were taken from each paraffin-embedded tissue block guided by hematoxylin and eosin (H&E) staining to ensure the collection of viable tumor tissue. These cores were subsequently allocated to a recipient paraffin block using a tissue arrayer (Beecher Instruments, USA). For each immunostaining, 3 µm thick slices were

cut from the TMA blocks using a microtome and placed on glass slides coated with 3-aminopropyltriethoxysilane to enhance tissue adhesion. Slides destined for our immunostaining were cut consecutively, to minimize the influence of tissue heterogeneity when comparing the expression of the different VEGF family members within each patient sample. In addition, we performed routine vimentin staining on test slides from each TMA to check for antigen integrity prior to further immunohistochemical analyses. To correct for differences in staining intensities between the different TMAs, control cores were present on each array containing tissue from various ovarian cancers (derived from a serous, endometrioid, mucinous, clear cell and undifferentiated tumor) as well as a benign ovarian cyst and normal endometrial and cervical tissue.

IMMUNOHISTOCHEMISTRY

TMA slides were deparaffinized using xylene and gradually rehydrated in a three-step ethanol dilution series. Heat-induced antigen retrieval was performed in 0.1 mM Tris/HCl (pH = 9.0) (VEGF-A) or 0.1 mM Tris/EDTA (pH = 9.0) (VEGF-B, VEGF-C, VEGF-D) using a 400 W rotary microwave. Endogenous peroxidase was blocked by incubating slides for 30 min with 0.3% H_2O_2 in phosphate-buffered saline (PBS: 6.4 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.14 mM NaCl, 2.7 mM KCl, pH = 7.2). After a wash-step, endogenous avidin and biotin activity was blocked using a commercially available blocking kit (Vector Laboratories, USA).

VEGF family members were stained using polyclonal rabbit anti-human antibodies directed against VEGF-A (sc-152), VEGF-B (sc-13083), VEGF-C (sc-9047) and VEGF-D (sc-13085) (Santa Cruz Biotechnology, USA). Primary antibodies were diluted 1:50 in PBS supplemented with 1% bovine serum albumin (BSA). Slides were incubated with the prediluted antibody for 60 min at room temperature and subsequently washed with Tris-buffered saline (TBS). Placental tissue sections were stained in parallel, providing a positive control. Primary antibodies were omitted in the incubation of negative control sections. A biotinylated goat anti-rabbit antibody (Dako, Denmark), diluted 1:300 in PBS supplemented with 1% BSA and 1% AB serum, was applied as a secondary antibody for 30 min. Following another TBS wash, slides were incubated for 30 min with a streptavidin-biotin/horseradish peroxidase complex (Dako) diluted 1:100 again in PBS supplemented with 1% BSA and 1% AB serum. Staining was visualized with 3,3'-diaminobenzidine (Sigma-Aldrich, USA) and hematoxylin counterstaining.

ANALYSIS OF IMMUNOSTAINED SLIDES

Following immunostaining, TMA slides were digitalized using the ScanScope GL system and the accompanying ImageScope v.9.1 software (Aperio, USA). Scoring of all cores was performed on the same monitor using the same screen settings, to ensure reproducibility.

The cytoplasmic staining intensity of cancer cells was categorized as absent (-), weak (-/+), moderate (+) or strong (++). Prior to the scoring of cores we selected reference cores for each expression category (**Figure 1**). Absence of staining was stringently defined as the total absence of any staining (*i.e.* comparable to the negative control sections). Internal positive controls were provided by the presence of (lymph)vascular endothelium in some of the cores, while non-tumorigenic stromal tissue (present in the benign ovarian cyst control) provided an internal negative control demonstrating tumor specificity of the antibodies. At least a quarter of the core area had to be evaluable to be designated an intensity score.

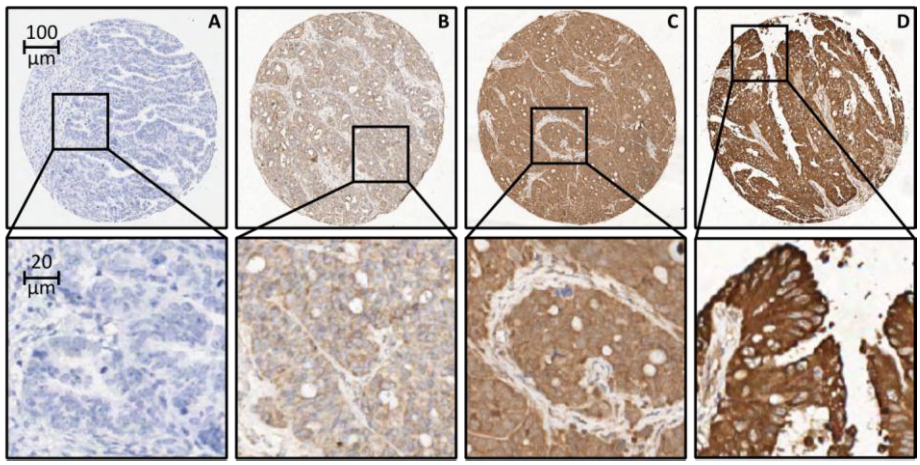

Figure 1. Reference cores from the ovarian cancer TMAs showing absent (A), weak (B), moderate (C) and strong (D) cytoplasmic expression of VEGF-A. Scale bars are indicated in the left panel. All cores depicted contain tissue from disseminated advanced-stage serous ovarian cancers. Similar staining intensities were used as references for the categorization of VEGF-B, VEGF-C and VEGF-D expression (reference cores not shown).

In case of tumor heterogeneity within one core, the intensity score was determined by the predominating staining intensity (> 50% of evaluable tumor tissue). Two observers (A.B. and K.H.) scored all TMA slides independently from each other and both were blinded to clinicopathological data (intra-observer variability: < 5%, inter-observer

variability: < 10%). A good correlation was found between digitalized scoring and conventional scoring using microscopy (κ = 0.72). Cores were scored randomly to avoid allocation bias of the four cores derived from one sample. Definitive staining intensities assigned to each sample were based on the most frequently observed intensity amongst at least two evaluable cores. In case of discordance, categorization was determined in dialogue with a gynecological pathologist.

STATISTICAL ANALYSIS

Correlations and differences between the expression levels of different VEGF family members or between primary cancers *versus* omental metastases were analyzed by determining Spearman's rank correlation coefficient and by performing Wilcoxon signed rank tests, respectively. Relations between immunohistochemical expression and common clinicopathological characteristics were analyzed using χ^2 tests and, where appropriate, Fisher's exact tests. For these analyses, all variables were dichotomized as indicated in the text. Disease stage was classified as early (FIGO I/II) or late stage (FIGO III/IV), and differentiation grade as low grade (grade I/II) or high grade (III). The prognostic value of VEGF family member expression was determined by univariate and multivariate Cox regression analyses. Only parameters that showed prognostic significance in univariate analysis were incorporated in the final multivariate model. Disease-specific overall survival (OS) as well as PFS was assessed, defined as the time from date of surgery until death due to ovarian cancer or progression/relapse of the disease, respectively. Statistics were performed using SPSS v.16.0 software (SPSS, USA). Differences were considered statistically significant at P < 0.05 (two-sided).

RESULTS

PATIENT CHARACTERISTICS

Patient characteristics are shown in **Table 1**. Median age at diagnosis was 58 years (range 16-89 years). Characteristics are comparable to those reported for other large cohorts of ovarian cancer patients. A total of 223 patients received chemotherapy, mostly consisting of carboplatin/paclitaxel (see **Table 1**). None of the patients received antiangiogenic drugs.

Table 1. Patient characteristics.

		All stages[a]		FIGO I		FIGO II		FIGO III		FIGO IV	
		N	*(%)*[b]	*N*	*(%)*	*N*	*(%)*	*N*	*(%)*	*N*	*(%)*
Origin tissue samples											
primary ovarian cancers		270		67		26		144		32	
omental metastases		112		0		1		90		21	
Histological subtype											
serous		147	(54%)	8	(12%)	12	(46%)	102	(71%)	25	(78%)
endometrioid		42	(16%)	21	(31%)	7	(27%)	11	(8%)	3	(9%)
mucinous		37	(14%)	26	(39%)	1	(4%)	9	(6%)	1	(3%)
clear cell		17	(6%)	6	(9%)	2	(8%)	7	(5%)	1	(3%)
other/unknown		27	(10%)	6	(9%)	4	(15%)	15	(10%)	2	(6%)
Differentiation grade											
grade I (well differentiated)		51	(19%)	31	(46%)	8	(31%)	12	(8%)	0	(0%)
grade II (moderately differentiated)		77	(29%)	31	(46%)	6	(23%)	33	(23%)	6	(19%)
grade III (poorly differentiated)		124	(46%)	2	(3%)	11	(43%)	88	(61%)	23	(72%)
unknown		18	(7%)	3	(5%)	1	(4%)	11	(8%)	3	(9%)
Tumor size prior to surgery											
small	(< 4 cm)	10	(4%)	3	(5%)	2	(8%)	4	(3%)	1	(3%)
moderate	(4-10 cm)	42	(16%)	12	(18%)	4	(15%)	22	(15%)	4	(13%)
large	(> 10 cm)	201	(74%)	47	(70%)	19	(73%)	109	(76%)	25	(78%)
unknown		17	(6%)	5	(8%)	1	(4%)	9	(6%)	2	(6%)
Residual tumor after primary surgery											
none (complete debulking)		117	(43%)	62	(93%)	18	(69%)	33	(23%)	3	(9%)
< 2 cm		38	(14%)	2	(3%)	3	(12%)	25	(17%)	8	(25%)
≥ 2 cm		94	(35%)	1	(2%)	3	(12%)	72	(50%)	18	(56%)
unknown		21	(8%)	2	(3%)	2	(8)	14	(10%)	3	(9%)
Chemotherapy											
no		40	(15%)								
yes	carboplatin/paclitaxel	93	(34%)								
	carboplatin/cyclophosphamide	81	(30%)								
	cisplatin/paclitaxel	8	(3%)								
	cisplatin/cyclophosphamide	8	(3%)								
	other	33	(12%)								
unknown		7	(3%)								

Abbreviations: N, number of patients/tissues; FIGO, International Federation of Gynecology and Obstetrics.

[a] Due to unknown disease stage, one case is missing in the data display per FIGO stage. It concerns a patient with a large grade II clear cell primary ovarian cancer, which was completely debulked.

[b] Cumulative percentages might not equal 100% due to rounding off.

VEGF FAMILY MEMBER EXPRESSION IN PRIMARY OVARIAN CANCERS

Thirteen TMAs were constructed and immunostained. Some tissue samples were excluded prior to analysis due to core loss during the staining procedures or due to lack of tumor tissue. Out of the 270 primary cancers, 244 could be analyzed for VEGF-A, 242 for VEGF-B, 237 for VEGF-C and 232 for VEGF-D expression. Staining intensities corresponded well amongst (and within) the four cores derived from one tissue sample, and staining heterogeneity was of minor influence. When more than two

evaluable cores were present, < 5% of cases needed reassessment before assigning a definitive staining intensity due to differences observed between cores.

All evaluable tissues expressed VEGF-A, VEGF-B, VEGF-C and VEGF-D to some degree (**Figure 2**). Of the primary cancers, 10% showed weak, 70% moderate and 20% strong VEGF-A expression. The immunohistochemical expression of VEGF-B was weak in 96% of the primary cancers, with the remaining 4% showing moderate expression. VEGF-C was expressed weakly by 59% and moderately by 40% of the primary ovarian cancers, while only three of the evaluable cancers showed strong expression (1%). Comparably, VEGF-D expression was weak in 45%, moderate in 54% and strong in 1% of the primary cancers.

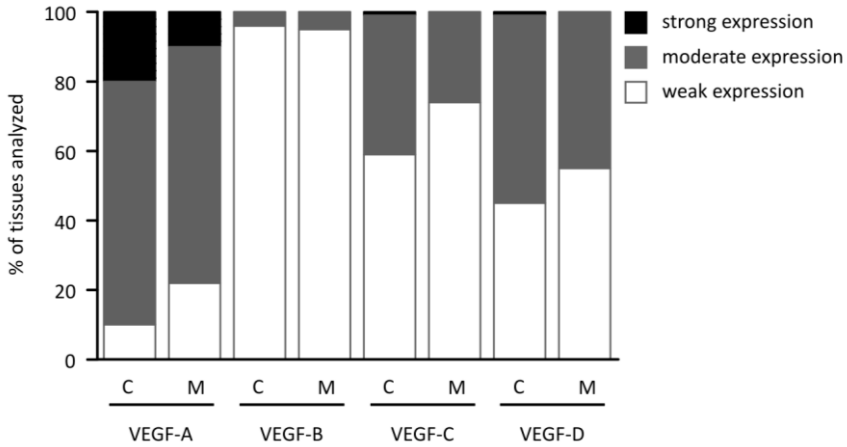

Figure 2. VEGF family member expression in ovarian cancer. The distribution of different immunohistochemical staining intensities in primary ovarian cancers (C) and omental metastases (M) is shown for VEGF-A, VEGF-B, VEGF-C and VEGF-D.

Simultaneous expression of VEGF-A and VEGF-C or VEGF-D is shown in **Figure 3**. VEGF-B was omitted in this analysis, given its practically invariable weak expression. Stronger expression of VEGF-A correlated with stronger expression of VEGF-C and VEGF-D (Spearman's correlation coefficient (ρ): 0.4, $P < 0.01$, both cases). As is represented in **Figure 3**, 38% of all primary ovarian cancers showed positivity (defined as moderate or strong expression) for both VEGF-A and VEGF-C, while more than half (54%) showed positivity for both VEGF-A and VEGF-D.

VEGF FAMILY MEMBER EXPRESSION IN OMENTAL METASTASES

Out of the 112 omental metastases available, 104 could be analyzed for VEGF-A, 102 for VEGF-B, 103 for VEGF-C and 101 for VEGF-D expression (**Figure 2**). VEGF-A was

expressed weakly in 22%, moderately in 68% and strongly in 10% of the metastases. For VEGF-B, 95% weak and 5% moderate expression was observed. VEGF-C expression was weak in 74% of the evaluable metastases and moderate in the remaining 26%, while VEGF-D showed 55% weak and 45% moderate staining. VEGF family member expression was comparable in primary tumors and their omental metastases (**Figure 4**). Slight expression differences between primary and metastatic disease most likely exemplify the dynamic nature of angiogenesis.

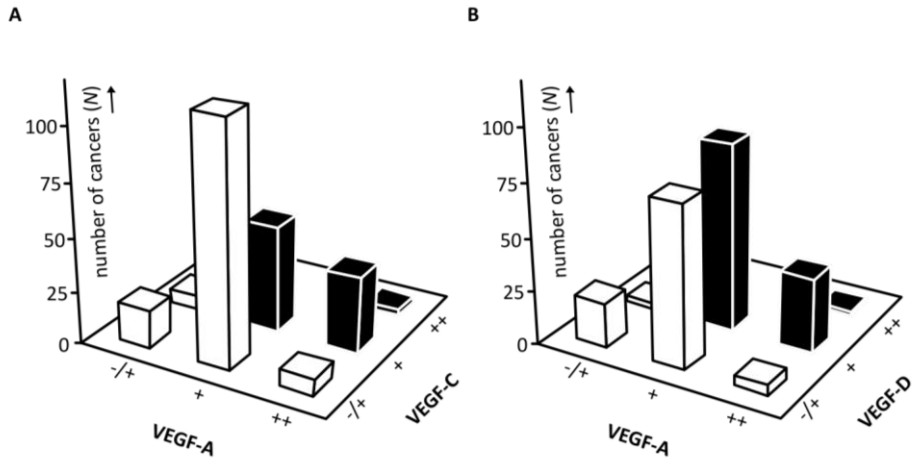

Figure 3. Simultaneous expression of VEGF-A and VEGF-C (A) or VEGF-D (B) in primary ovarian cancers. Combined expression data were available in 235 and 230 cancers, respectively. Black bars represent the fraction of patients with positivity (*i.e.* moderate or strong expression) for both cytokines, showing positivity for multiple proangiogenic VEGF family members in many primary ovarian cancers. In addition, positivity for VEGF-C and VEGF-D was almost exclusively observed in cancers that are positive for VEGF-A.

VEGF FAMILY MEMBER EXPRESSION IN RELATION TO CLINICOPATHOLOGICAL CHARACTERISTICS

The various histological tumor subtypes were differentially distributed between FIGO stages and differentiation grades (see **Table 1**). Since different tumor histology represents distinct biological behavior, apart from general correlations between clinicopathological characteristics and VEGF family member expression, analyses were also performed per histological subgroup (distinguishing between serous, endometrioid, mucinous, clear cell and other histology). Immunohistochemical expression was dichotomized as either negative (absent and weak expression) or positive (moderate and strong expression). No associations were observed between primary cancer expression of any of the VEGF family members and FIGO stage, histological subtype, patient age (dichotomized by median age) and volume of ascites

(**Table 2**). An inverse relationship was observed for VEGF-A, VEGF-C and VEGF-D with differentiation grade. Subgroup analysis revealed that this relation is only present in mucinous cancers ($P < 0.01$ using Fisher's exact tests), which is likely caused by compression of the cytoplasm in better differentiated mucinous cancers (due to the presence of a mucous vacuole). Expression of VEGF family members in the omental metastases was not associated with any of the clinicopathological characteristics mentioned (data not shown).

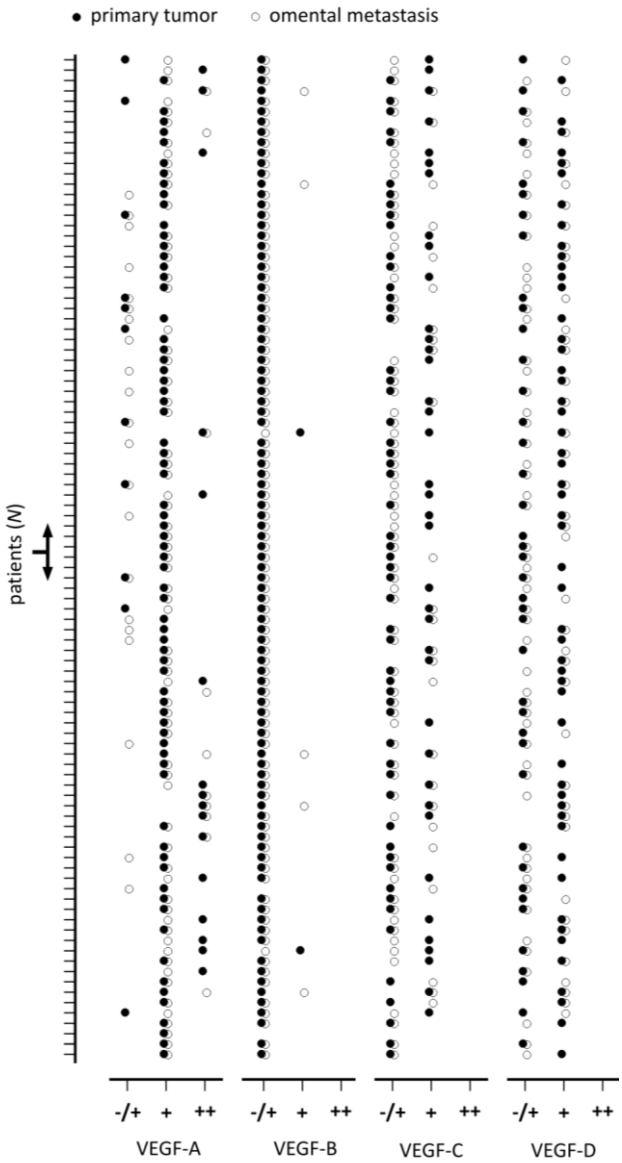

Figure 4. Expression of VEGF family members in primary cancers and related omental metastases. Paired expression data were present in 97 patients for VEGF-A, 95 patients for VEGF-B, 93 patients for VEGF-C and 92 patients for VEGF-D. Compared to their cancers of origin, expression in omental metastases was similar in 65% of cases for VEGF-A, 93% for VEGF-B, 62% for VEGF-C and 60% for VEGF-D. In cases of discordant expression between primary and metastatic tissue, the difference is limited: strong *versus* weak expression or vice versa is never seen. Omental metastases tend to show less expression compared to their primary cancers: lower metastases expression was observed in 74% (VEGF-A), 68% (VEGF-C) and 68% (VEGF-D) of cases in which tumor and metastases expression differed ($P < 0.05$).

Table 2. Relations between protein expression and clinicopathological characteristics.

	Expression in primary cancers											
	VEGF-A			VEGF-B			VEGF-C			VEGF-D		
Variable	*Neg*	*Pos*	*P*	*Neg*	*Pos*	*P*	*Neg*	*Pos*	*P*	*Neg*	*Pos*	*P*
Stage												
early	7	78	NS	78	5	NS[a]	42	40	NS	28	47	NS
late	18	140		153	5		97	57		76	80	
Grade												
low	6	107	**.02**	104	7	NS[a]	50	59	**.00**	34	67	**.00**
high	17	96		112	2		80	31		61	53	
Ascites												
< 1L	13	83	NS	89	5	NS[a]	54	39	NS	42	46	NS
≥ 1L	7	68		73	3		45	27		33	42	
Age												
< 58 years	11	109	NS	115	4	NS[a]	68	51	NS	51	63	NS
≥ 58 years	14	110		117	6		72	46		53	65	
Histology[b]												
serous	9	123	NS	130	3	NS	82	45	NS	66	63	NS
endometrioid	7	33		37	1		19	20		17	19	
mucinous	5	29		30	4		17	17		7	24	
clear cell	2	13		13	1		9	6		6	8	
other	2	21		22	1		13	9		8	14	

Abbreviations: NS, not statistically significant ($P \geq 0.05$).

[a] Fisher's exact test was used instead of Chi-square analysis to determine statistical significance, due to expected cell counts of less than 5 in at least one cell.

[b] Serous cancers were also compared to all other subtypes (grouped), demonstrating no significant relations between histology and VEGF family member expression.

PROGNOSTIC SIGNIFICANCE OF VEGF FAMILY MEMBER EXPRESSION

The prognostic significance of the different VEGF family members was determined in our cohort of ovarian cancer patients, in relation to known prognostic factors (**Table 3**). Multivariate survival analysis demonstrated no independent prognostic value of any of the VEGF family members expressed in primary cancers or metastases.

DISCUSSION

This is the first study in which the expression of VEGF-A, VEGF-B, VEGF-C and VEGF-D were investigated together in primary and metastatic ovarian cancer. Our data demonstrate that multiple proangiogenic VEGF family members are often expressed simultaneously in these cancers.

Previous studies investigating the expression of VEGF-A in ovarian cancer have reported varying results, in general in much smaller series.[10-16] While strong immunohistochemical expression of VEGF-A has previously been observed in up to

Table 3. Results from univariate and multivariate survival analysis.

	Cox regression analysis					
	Univariate			Multivariate		
	HR	*95% CI*	*P*	*HR*	*95% CI*	*P*
Progression-free survival						
Late stage (FIGO III/IV)	5.23	3.43-7.98	**.00**	2.63	1.49-4.64	**.00**
Residual tumor (any)	4.57	3.16-6.61	**.00**	2.47	1.53-3.99	**.00**
High grade (grade III)	2.85	2.02-4.02	**.00**	1.44	0.94-2.21	NS[a]
Age (≥ 58 years)	1.48	1.07-2.04	**.02**	1.19	0.82-1.73	NS
Primary cancer expression						
VEGF-A positive	0.81	0.47-1.42	NS			
VEGF-B positive	0.10	0.01-0.69	**.02**	0.15	0.02-1.07	NS[a]
VEGF-C positive	0.64	0.44-0.91	**.01**	0.95	0.64-1.42	NS
VEGF-D positive	0.73	0.52-1.03	NS			
Metastases expression						
VEGF-A positive	0.92	0.53-1.60	NS			
VEGF-B positive	0.75	0.30-1.86	NS			
VEGF-C positive	1.25	0.76-2.04	NS			
VEGF-D positive	0.77	0.48-1.22	NS			
Overall survival[b]						
Late stage (FIGO III/IV)	7.91	4.54-13.80	**.00**	3.21	1.54-6.70	**.00**
Residual tumor (any)	5.86	3.81-9.01	**.00**	2.73	1.56-4.79	**.00**
High grade (grade III)	3.44	2.37-4.99	**.00**	1.51	0.96-2.38	NS[a]
Age (≥ 58 years)	1.90	1.35-2.69	**.00**	1.37	0.92-2.03	NS
Primary cancer expression						
VEGF-A positive	1.21	0.59-2.48	NS			
VEGF-B positive	0.11	0.02-0.81	**.03**	0.19	0.03-1.39	NS[a]
VEGF-C positive	0.59	0.40-0.87	**.01**	0.86	0.54-1.37	NS
VEGF-D positive	0.65	0.46-0.94	**.02**	0.86	0.56-1.32	NS
Metastases expression						
VEGF-A positive	1.02	0.57-1.82	NS			
VEGF-B positive	0.54	0.19-1.48	NS			
VEGF-C positive	1.32	0.81-2.15	NS			
VEGF-D positive	1.30	0.83-2.02	NS			

Abbreviations: HR, hazard ratio; 95% CI, 95% confidence interval; NS, not statistically significant ($P \geq 0.05$); FIGO, International Federation of Gynecology and Obstetrics.

[a] Borderline statistical significance: *P*-values ranging between 0.05 and 0.10.

[b] Disease-specific overall survival was defined as the time between initial surgery and death due to ovarian cancer.

90% of primary ovarian cancers[11-14], others described lack of expression in 9-50%.[10,15,16] The different immunohistochemical expression levels that have been reported likely reflect the divergent cut-off values and scoring criteria used. We observed omnipresent VEGF-A immunostaining, which is supported by the omnipresence of VEGF-A mRNA found in PCR studies in ovarian cancers.[17]

Much less is known about the expression of the other VEGF family members in ovarian cancer. To our knowledge, VEGF-B protein expression has not been previously investigated in ovarian cancer. Our results demonstrate a generalized weak expression of VEGF-B in ovarian cancer. However, many primary ovarian cancers did exhibit immunohistochemical positivity for VEGF-C and VEGF-D. Smaller previous studies, often discriminating between low and high immunohistochemical positivity, have reported high expression for VEGF-C in 32-49% and VEGF-D in 25-27% of primary ovarian cancers.[12,18-20] A study in which VEGF-C and VEGF-D expression were classified in a similar manner compared to our study reported moderate or strong expression of these angiokines in almost two-thirds of the 59 ovarian cancers analyzed, while negative staining was only observed in 0-7% of cases.[20] In addition, it has been demonstrated that VEGF-C and VEGF-D are both strongly upregulated in ovarian cancer *versus* non-malignant ovarian disease[20], which has also been described for VEGF-A.[11,21]

Although its key ligand is VEGF-A, VEGFR2 can also be activated by proteolytically cleaved forms of VEGF-C and VEGF-D.[2] Moreover, animal studies have implied VEGFR3 signaling (activated by VEGF-C and VEGF-D) in angiogenesis.[7,8] Inhibition of VEGFR3 with receptor-blocking antibodies impairs angiogenesis *in vivo* (both in a mouse retina model as well as in tumor-bearing xenografts) and, conversely, VEGFR3 signaling can sustain angiogenesis even in the presence of VEGFR2 inhibition.[8] Interestingly, induction of VEGFR3 expression on the vascular endothelium has been shown upon signaling by the VEGF-A-VEGFR2 system. VEGFR3 induction has also been described in paraffin-embedded tissue from ovarian cancer patients, showing 6-fold higher VEGFR3 expression by endothelial cells neighboring ovarian cancer cells when compared to endothelium associated with benign ovarian masses.[20] These findings indicate a clear role for both VEGFR2 and VEGFR3 in ovarian cancer angiogenesis, and in combination with the substantial expression of VEGF-C and VEGF-D observed in our study, suggest that VEGF-C and VEGF-D can be relevant targets for antiangiogenic therapy in ovarian cancer.

We observed marked expression of VEGF-C or VEGF-D in more than 40% of the cancers which also express VEGF-A. Translating this to patient treatment, solely blocking VEGF-A with bevacizumab might not provide adequate angiogenesis inhibition due to remaining angiogenic signaling by VEGF-C and VEGF-D. Therefore, patients that express multiple VEGF family members might benefit more from antiangiogenic treatment with VEGFR-targeted TKIs active against both VEGFR2 and VEGFR3, such as

sorafenib or cediranib. Alas, none of the clinical trials to date with angiogenesis inhibitors in ovarian cancer have assessed the expression of different VEGF family members to compare between responders *versus* non-responders.

Understanding the molecular mechanisms underlying upfront and acquired resistance to antiangiogenic therapy can facilitate patient selection strategies. Recently, prolonged *in vitro* exposure of colorectal cancer cells to bevacizumab was shown to induce compensatory secretion of several VEGF family members, with a strong induction seen for VEGF-C.[22] Induction of VEGF-C and VEGF-D was also described in glioma cells incubated in the presence of bevacizumab.[23] In addition, bevacizumab was shown to increase endothelial cell responsiveness to stimulation by VEGF-C and VEGF-D, resulting in their proliferation, migration and tube formation despite effective VEGF-A blockade.[23] These results highlight the potential role for VEGF-C and VEGF-D in bevacizumab resistance.

While some studies indicate that stronger immunohistochemical expression of VEGF-A, VEGF-C or VEGF-D independently predicts worse outcome[10,18-20,24], other studies show no prognostic role of their expression in ovarian cancer.[11,13,14,25] Our data show no prognostic significance for any of the VEGF family members analyzed in a large cohort. Studies in which the expression of VEGF family members is of prognostic significance generally report hazard ratios that are much smaller than those found for disease stage, grade or residual disease after debulking. Taken together, VEGF family member expression does not seem to provide a clinically reliable prognostic indicator and seems of no additional value to well-established prognostic factors.

In summary, we demonstrate that VEGF-A, VEGF-C and VEGF-D are widely and often simultaneously expressed in ovarian cancer. Since signaling by all three of these VEGF family members is involved in angiogenesis and can be targeted by different classes of antiangiogenic drugs, analyzing their simultaneous expression could provide a useful tool in patient-tailoring antiangiogenic therapy in ovarian cancer.

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CHAPTER 4

^{89}ZR-BEVACIZUMAB PET AS AN EARLY BIOMARKER FOR THE ANTIANGIOGENIC EFFECT OF EVEROLIMUS TREATMENT IN AN OVARIAN CANCER XENOGRAFT MODEL

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Revised and resubmitted for publication

ABSTRACT

BACKGROUND The mammalian target of rapamycin (mTOR) pathway is frequently activated in ovarian cancers. mTOR inhibitors, like everolimus, can reduce vascular endothelial growth factor-A (VEGF-A) production by cancer cells. We investigated whether early everolimus treatment effects could be monitored by positron emission tomography (PET) with ^{89}Zr-bevacizumab.

METHODS The effect of everolimus on VEGF-A secretion was determined in a panel of human ovarian cancer cell lines and in A2780^{luc+} ovarian cancer cells xenografted subcutaneously in BALB/c mice. Mice received daily 10 mg/kg everolimus intraperitoneally for 14 days. PET scans with the tracer ^{89}Zr-labeled bevacizumab were performed before and after treatment. *Ex vivo* ^{89}Zr-bevacizumab biodistribution and correlative tissue analyses were performed. Tumor VEGF-A levels were measured with ELISA and mean vascular density (MVD) was determined with immunohistochemistry.

RESULTS Everolimus treatment reduced VEGF-A levels in the supernatant of all cell lines. Everolimus lowered ^{89}Zr-bevacizumab tumor uptake by 21.7 ± 4.0% (SUV_{mean} 2.3 ± 0.2 *versus* 2.9 ± 0.2, $P < 0.01$). *Ex vivo* biodistribution also demonstrated lower tracer uptake in the tumors of treated compared to control animals (7.8 ± 0.8 %ID/g *versus* 14.0 ± 1.7 %ID/g, $P < 0.01$), while no differences were observed for other tissues. This coincided with lower VEGF-A protein levels in tumor lysates in treated *versus* untreated tumors ($P = 0.04$) and reduced MVD ($P < 0.01$).

CONCLUSION Tumor VEGF-A levels are decreased by everolimus. ^{89}Zr-bevacizumab PET could be used to monitor tumor VEGF-A levels as an early biomarker of the antiangiogenic effect of mTOR inhibitor therapy.

INTRODUCTION

Ovarian cancer patients often present with advanced-stage disease and develop resistance to conventional (platinum-based) chemotherapy during the course of treatment, resulting in a poor 30% 5-year survival rate.[1] To improve ovarian cancer prognosis, there is a clear need for additional therapeutic options. Since ovarian cancers are often extensively vascularized and overexpress proangiogenic factors like vascular endothelial growth factor-A (VEGF-A), angiogenesis inhibition has been studied as a therapeutic strategy. Phase II and III trials with the VEGF-A-neutralizing antibody bevacizumab or with VEGF receptor (VEGFR)-targeted tyrosine kinase inhibitors (TKI), targeting vascular endothelial cells, have shown antitumor activity in a subgroup of patients.

The kinase mammalian target of rapamycin (mTOR) is a potential alternative target for antiangiogenic therapy. The mTOR pathway is activated in 64-85% of ovarian cancers.[2,3] mTOR enhances translational efficacy of mRNAs that contain intricate 5'-untranslated regions (UTRs). These regions encode oncogenic proteins like hypoxia-inducible factor (HIF) and VEGF-A.[4,5] By reducing the synthesis of these proteins by tumor cells, mTOR inhibitors have distinct antiangiogenic effects compared to classical antiangiogenic drugs like bevacizumab.[6]

In transgenic (orthotopic) and xenograft mouse models, treatment with the mTOR inhibitor everolimus delayed tumor development, reduced tumor burden and prolonged survival.[7-10] mTOR inhibition reduced VEGF-A expression and diminished vascularization in murine (xenograft) ovarian cancers.[7,8] This was even more pronounced when combined with bevacizumab.[11]

In a phase II trial, 60 patients with persistent or recurrent ovarian cancer received weekly temsirolimus.[12] A partial tumor response was observed in 9.3% of the patients, with disease stabilization in a further 40.7%.[12] This underscores the importance of discovering biomarkers for upfront patient selection or early response prediction. However, no biomarkers are currently established for predicting everolimus or VEGF(R)-targeted drug efficacy in ovarian cancer.

Whole-body positron emission tomography (PET) can potentially address this issue. It offers the possibility for longitudinal *in vivo* monitoring of tumor biology and changes herein upon treatment. In order to visualize antiangiogenic drug efficacy, we generated radiolabeled bevacizumab which proved able to non-invasively detect tumor VEGF-A levels in animal models and patients.[13-15]

The aim of this study was to analyze the effect of everolimus on ovarian cancer VEGF-A secretion *in vitro* and *in vivo*, and the potential of serially measuring tumor VEGF-A levels with ^{89}Zr-bevacizumab PET as an early predictive biomarker for everolimus antiangiogenic efficacy in an ovarian cancer xenograft model.

METHODS

CELL LINES

The human ovarian cancer cell line A2780 was kindly provided by Dr TC Hamilton (Fox Chase Cancer Center). The luciferase transfected subline A2780^{luc+} was developed as described earlier.[16] SKOV-3 and TOV-21G ovarian cancer cell lines were obtained from the American Type Culture Collection (ATCC). A2780 and A2780^{luc+} cells were cultured in RPMI1640, supplemented with 10% fetal calf serum (FCS, Bodinco BV) and 2 mM L-glutamine (Invitrogen), SKOV-3 in Dulbecco's Modified Eagles Medium (DMEM) with 4.5 g/L glucose, supplemented with 10% FCS, and TOV-21G cells in Ham-F12 and DMEM (1:1), supplemented with 10% FCS. All culture media were purchased from Invitrogen. All cell lines were cultured at 37 °C in a fully humidified atmosphere containing 5% CO_2.

For *in vitro* experiments, cells were harvested with trypsin after a single wash with phosphate-buffered saline (PBS: 6.4 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.14 mM NaCl, 2.7 mM KCl, pH = 7.2) and seeded in 6-well plates (A2780: 31,500 cells/cm^2, SKOV-3: 21,000 cells/cm^2, TOV-21G: 52,500 cells/cm^2). The next day, everolimus was added in fresh medium (1.5 mL/well) to reach final concentrations of 0-100 nM.

COMPOUNDS

Everolimus was obtained from LC Laboratories (E-4040, stored at -20 °C). For *in vitro* use, everolimus was dissolved in dimethyl sulfoxide (DMSO) at 10 mM stock concentration stored at -20 °C. For *in vivo* use, everolimus was dissolved in DMSO and formulated in a stable suspension with PBS containing 0.5% Tween-80 (Sigma-Aldrich). Mice received 10 mg/kg everolimus daily via intraperitoneal injection (10 mL/kg). The everolimus dosage used is based on previous efficacy studies in xenografted mice, where 5-10 mg/kg daily resulted in unbound (active) everolimus plasma levels comparable to those reached in humans receiving 5-10 mg daily due to higher plasma protein binding and shorter $T_{1/2}$ values in mice.[6,17-20]

WESTERN BLOTTING

Total and phosphorylated protein expression of mTOR target proteins was measured by Western blotting. After 24 hours everolimus exposure, adherent cell layers were washed three times with ice-cold PBS and total cellular protein content was extracted with mammalian protein extraction reagent (M-PER, Thermo Scientific). Protease and phosphatase inhibitors (Thermo Scientific) were added (1:100). Protein yield was determined with the Bradford assay.[21] Samples were diluted 1:1 with sodium dodecyl sulphate (SDS) sample buffer, containing 125 mM Tris-HCl, 2% SDS, 10% glycerol, 0.001% bromophenol blue and 10% β-mercaptoethanol, and boiled for 5-10 minutes before storage at -20 °C. Proteins (20-30 µg) were separated on SDS-polyacrylamide gels and transferred onto polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore) by wet blotting.[22] Primary antibodies were purchased from Cell Signaling, recognizing epitopes on p70S6K (#2708), phosho-p70S6K (Thr389) (#9206), S6 (#2217), phospho-S6 (Ser235/236) (#4856), 4E-BP1 (#9644) and phospho-4E-BP1 (Thr70) (#9455). Anti-β actin antibody (BP Biomedicals) served to assure equal protein loading. Membranes were incubated with horseradish peroxidase-labeled secondary antibodies (DAKO) for 1 hour at room temperature. Protein bands were visualized with chemiluminescence using Lumi-Light^{plus} (Roche Diagnostics).

VEGF-A ELISA

After 24 hours incubation with everolimus, cell culture supernatant was removed and centrifuged for 10-15 minutes at 450 x g, to remove any residual cells or cell remnants, and subsequently stored in aliquots at -20 °C until VEGF-A levels were measured with VEGF Quantikine enzyme-linked immunosorbent assay (ELISA) kits (DVE00, R&D Systems). To correct for experimental differences, cell counting was performed in parallel for each sample using conventional counting grids and trypan blue staining.

For *in vivo* studies, three random cores were die-cut from frozen tumor samples and lysed using M-PER.[14] In these whole-tumor protein lysates, VEGF-A levels were assayed with ELISA kits and total protein content with Bradford assays as described in the above.

XENOGRAFT MODEL

All animal experiments were approved by the animal experiments committee of the University of Groningen. Animal studies were performed in male nude BALB/c mice (BALB/cOlaHsd-*Foxn1*^{nu}, Harlan). Animals were allowed to feed *ad libitum*. Experiments

were performed under isoflurane inhalation anesthesia (induction 3%, maintenance 1.5%).

Mice (*N* = 10) were xenografted subcutaneously in the flank with 5 x 10^6 A2780^{luc+} cells dissolved in 0.3 mL of a 1:1 mixture of PBS and Matrigel (BD Biosciences). To ensure tumor viability, bioluminescence imaging was performed using the IVIS Spectrum system (Caliper Life Sciences). D-luciferin (150 mg/kg, purchased from Xenogen) was reconstituted in PBS and injected intraperitoneally 20 minutes before image acquisition. Twelve days after tumor inoculation, when the tumor diameter measured 6-8 mm (~300 mm^3), a baseline 6-day microPET scan sequence was performed to determine pretreatment ^{89}Zr-bevacizumab tracer uptake. Daily everolimus treatment (10 mg/kg intraperitoneal administration) was started immediately after completing the baseline scan sequence and continued for 14 days. On day 9 during everolimus treatment, a second 6-day scan sequence was initiated after ^{89}Zr-bevacizumab injection. Animals that received everolimus (*N* = 5) were sacrificed immediately after acquiring the posttreatment scans.

To allow comparison between treated *versus* untreated tumors, a separate group of mice (*N* = 5, control group) was sacrificed after the baseline scans. Mice xenografted with A2780^{luc+} cells develop rapidly growing tumors, with a doubling time of 3-6 days.[14] Therefore, control animals cannot be maintained for the same duration as the treatment group, since tumors will grow beyond tolerable sizes. To obtain data on tumor volumes, regular caliper measurements were performed as described previously.[15] Tumor growth curves were related to control data from untreated A2780^{luc+} tumor-bearing mice obtained earlier.[15]

MICROPET IMAGING

Bevacizumab conjugation and labeling were performed as described previously.[13] ^{89}Zr-bevacizumab (± 5 MBq, ± 30 µg) was injected into the penile vein. Animals were imaged using a MicroPET Focus 220 rodent scanner (CTI/Siemens). Static images were obtained (15-45 minutes acquisition) immediately after tracer injection and at 144 hours post-injection, as ^{89}Zr-bevacizumab tumor uptake in this animal model was shown to be optimal at 144 hours after tracer injection.[13,14] Images were analyzed and quantified using AMIDE software (version 0.9.1, Stanford University).[23] Imaging data are presented as the mean standardized uptake value (SUV_{mean}).[15]

BIODISTRIBUTION

Organs and tissues were excised, rinsed of residual blood and weighed. Tissues were counted for radioactivity in a calibrated well-type LKB-1282-Compu-gamma system (LKB Wallac), together with primed standards to correct for physical decay. *Ex vivo* tissue activity is expressed as the percentage of the injected dose per gram of tissue (%ID/g). Harvested tumors were divided, and partly paraffin-embedded and partly frozen at -80 °C for further *ex vivo* analysis.

IMMUNOHISTOCHEMISTRY

Slices (3 µm thick) were cut from paraffin-embedded tumor sections using a microtome and placed on 3-aminopropyltriethoxysilane-coated glass slides. Heat-induced antigen retrieval was performed in 10 mM citrate buffer (S6, p-S6) or Tris/EDTA (Ki67) using a 400 W rotary microwave, or by using Proteinase K (vWF) at room temperature. Endogenous peroxidase was blocked by 30 minutes incubation with 0.3% H_2O_2 in PBS. Endogenous avidin/biotin activity was blocked using a commercially available blocking kit (Vector Laboratories). Slides were incubated with primary antibodies detecting human S6 and p-S6 (Cell Signaling, #2217 and #2211) and Ki67 (Dako, 1:350). In addition, slides were stained with anti-Von Willebrand factor antibody (vWF) (Dako, 1:250) to determine the mean vascular density (MVD).[15] Staining was visualized after incubation with biotinylated or peroxidase-bound secondary antibodies (Dako) using, when necessary, the streptavidin-biotin/horseradish peroxidase complex (Dako) and 3,3'-diaminobenzidine (Sigma-Aldrich). Hematoxylin counterstaining was applied routinely and hematoxylin & eosin (H&E) staining served to analyze tissue viability and morphology.

Stainings were quantified by evaluation of 10 high-power fields (400x magnification) using a calibrated grid, counting positive and negative cells (S6 and p-S6, cytoplasmic staining) or nuclei (Ki67). Data is expressed as the percentage of positive cells. MVD was determined by counting the number of vessels within at least 3 angiogenic hot-spots as described previously.[15] Photographs were acquired by digitalized scanning of slides using the NanoZoomer 2.0-HT multi-slide scanner (Hamamatsu). To calculate tumor viability, acquired images from H&E stained slides were analyzed using NanoZoomer Digital Pathology (NDP) viewer software (Hamamatsu). Using the freehand surface area tool, areas with non-viable tumor tissue were delineated within fixed-size squares overlaying the surface area of the slide.

STATISTICAL ANALYSES

Data are presented as mean ± standard deviation (SD). For *in vitro* experiments, different experimental conditions were compared using unpaired Student's *t*-tests. Comparison of ^{89}Zr-bevacizumab uptake before and after everolimus treatment was performed using the paired t-test, while unpaired testing was used for *ex vivo* analyses (comparing control *versus* treatment group data). Statistical analyses were performed using Prism v.5 (GraphPad). A *P*-value of < 0.05 (two-tailed) was considered significant.

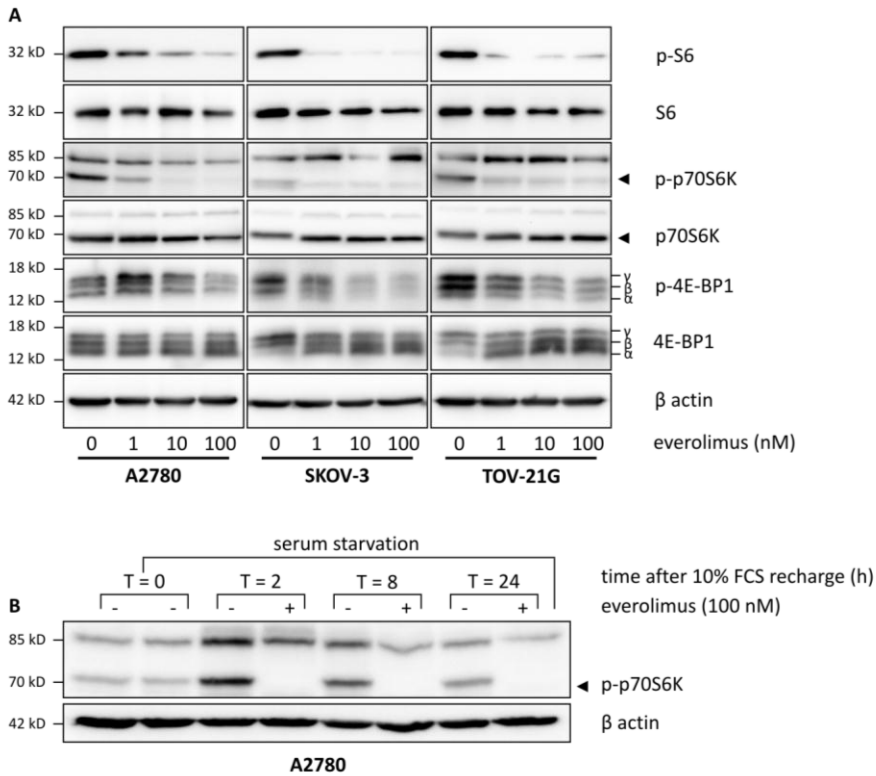


Figure 1. (A) A2780, SKOV-3 and TOV-21G cells were treated for 24 hours with everolimus at the indicated concentrations. Western blotting showed that phosphorylation of downstream mTOR target proteins S6, p70S6K and 4E-BP1 was effectively reduced in all cell lines at all concentrations used. The upper band in the (p-)p70S6K blots is a consequence of analogous binding to p85S6K, which is another S6K splice variant. The α, β and γ isoforms of 4E-BP1 reflect differential phosphorylation, with the γ isoform being most extensively phosphorylated. β actin was used as a loading control. (B) A2780 cells were serum starved overnight before everolimus (100 nM) was added to the culture medium. After 30 minutes of pre-incubation with everolimus, FCS was added for the indicated timepoints. As shown by the lack of p70S6K phosphorylation, mTOR is already inhibited shortly after drug exposure and inhibition persisted throughout the whole incubation period.

RESULTS

mTOR INHIBITION BY EVEROLIMUS REDUCES OVARIAN CANCER VEGF-A SECRETION IN VITRO

Target inhibition by everolimus was shown by reduced phosphorylation of p70S6K, S6 and 4E-BP1 in all cell lines tested (**Figure 1A**). mTOR inhibition occurred within 2 hours upon everolimus exposure (**Figure 1B**).

Everolimus reduced VEGF-A secretion by all cell lines ($P < 0.05$ at all concentrations used, compared to untreated controls) (**Figure 2**). The maximum effect was achieved at relatively low concentrations of everolimus (10 nM). After 24 hours drug exposure, VEGF-A levels in the culture supernatant were reduced by 57 ± 7% in A2780, 42 ± 15% in SKOV-3 and 38 ± 3% in TOV-21G cells compared to untreated cells. Cell counting experiments revealed no effect on cell number or viability after 24 hours everolimus incubation (**Supplementary Figure S1**).

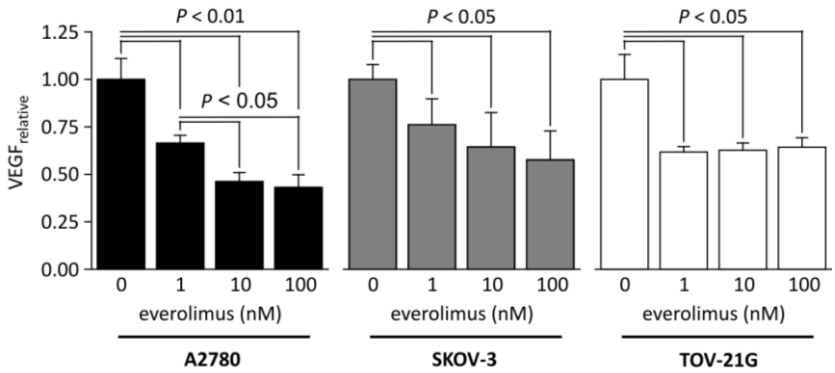


Figure 2. Everolimus reduces VEGF-A secretion in a panel of three ovarian cancer cell lines. Cells were exposed for 24 hours at the indicated concentrations of everolimus. VEGF-A levels (measured in pg/mL supernatant) are expressed relative to those measured in the supernatant of untreated cells, corrected for cell number. VEGF-A levels were lower at all concentrations used ($P < 0.05$).

EVEROLIMUS REDUCES ^{89}ZR-BEVACIZUMAB TUMOR UPTAKE IN XENOGRAFTED MICE

^{89}Zr-bevacizumab tracer uptake was homogeneous within the tumors analyzed at each timepoint. Everolimus decreased ^{89}Zr-bevacizumab tumor uptake compared to pretreatment (baseline) scans in all animals. **Figure 3A** shows representative PET images. In pretreatment scans, the SUV_{mean} was 2.9 ± 0.2, compared to 2.3 ± 0.2 in posttreatment scans after 2 weeks of everolimus treatment (21.7 ± 4.0% decrease in tracer uptake, $P = 0.0005$) (**Figure 3B**).

Analogous, *ex vivo* biodistribution experiments demonstrated a lower ^{89}Zr-bevacizumab tumor uptake in everolimus treated animals *versus* control animals (7.8 ±

0.8 %ID/g *versus* 14.0 ± 1.7 %ID/g, $P < 0.01$; **Figure 3C**). No differences in ^{89}Zr-bevacizumab uptake were observed for non-tumor tissues between treated and untreated animals (**Supplementary Figure S2**).

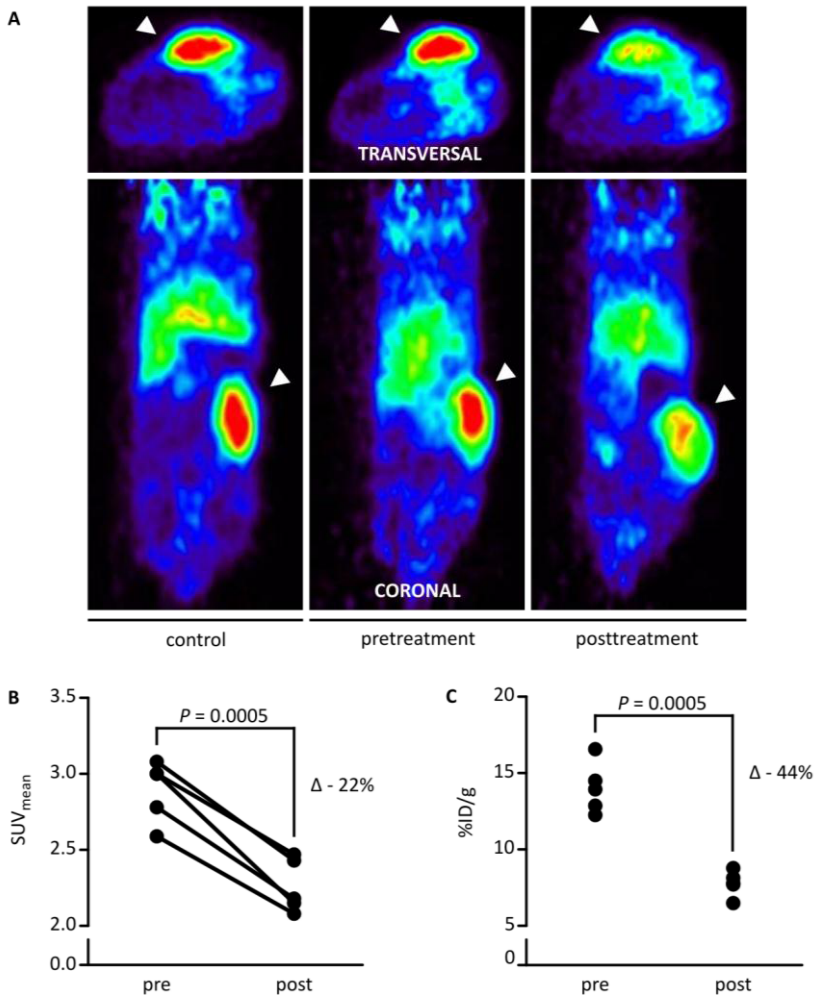


Figure 3. (A) Representative transversal and coronal ^{89}Zr-bevacizumab microPET images of A2780^{luc+} xenografted mice in the control and treatment group. Pre- and posttreatment images are from the same animal. Tumors are indicated by arrowheads. Scans were acquired with the animals lying sideways with the inoculated left flank upwards. Tracer uptake in the tumor is clearly visible, as well as some aspecific liver uptake. (B) MicroPET quantification in individual everolimus treated animals demonstrated a clear reduction in the SUV_{mean} compared to pretreatment scans. A 22 ± 4.0% decrease in tracer uptake was observed (P = 0.0005). (C) *Ex vivo* biodistribution was performed in control and treated animals directly after obtaining the final ^{89}Zr-bevacizumab PET scans. Tracer uptake is expressed as percentage of injected dose per g tissue (%ID/g). Compared to tumors derived from control animals, everolimus treated animals show lower tumor uptake by 44% (P = 0.0005).

During the 14-day treatment period, tumor volumes in mice receiving everolimus increased to a maximum of 135 ± 17% compared to the start of treatment (**Figure 4**). Tumor growth during everolimus treatment was slower compared to historical controls (untreated A2780^{luc+} xenograft-bearing mice), which showed a similar increase in tumor volume already at day 3 after baseline scans.[15]

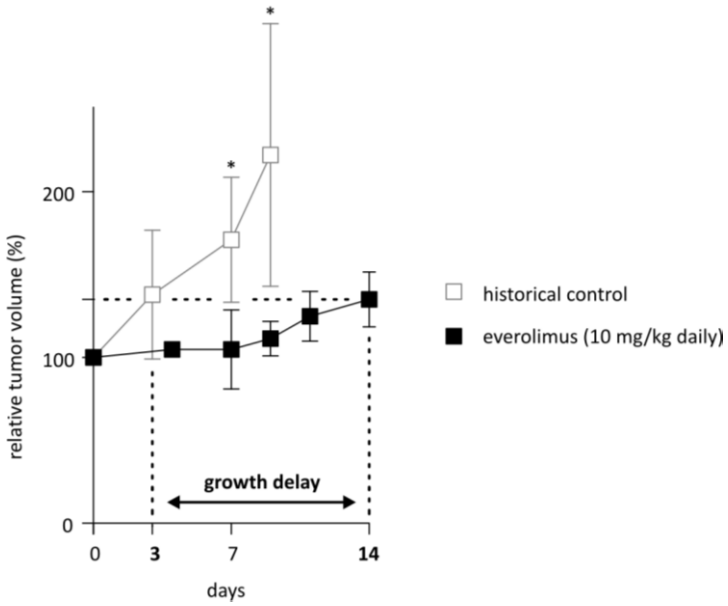


Figure 4. Tumor size determined by caliper measurements in everolimus treated animals compared to historical controls (untreated A2780^{luc+} xenograft-bearing mice). The point of reference is set at day 0, the last day of the baseline scanning sequence after which everolimus administration was started. After 7 days of everolimus treatment, the tumors were smaller in treated animals *versus* controls. Control animals had to be terminated at day 9 because of excessive tumor outgrowth.

Decreased ^{89}Zr-bevacizumab tumor uptake corresponds with reduced tumor VEGF-A levels and reduced vascularity after everolimus treatment

Everolimus treatment resulted in effective target inhibition as the expression of phosphorylated S6 was clearly reduced in treated tumors *versus* tumors obtained from control animals after baseline scanning, while total S6 expression was not affected (**Figure 5A**). Morphologic analysis of H&E stained slides revealed no difference in tumor viability between treated and control tumors (**Figure 5B**). After 2 weeks of everolimus treatment the Ki67-based proliferation index was lower in treated (30.21 ± 1.76%) compared to control tumors (50.86 ± 1.46%, $P < 0.0001$) (**Figure 5C**).

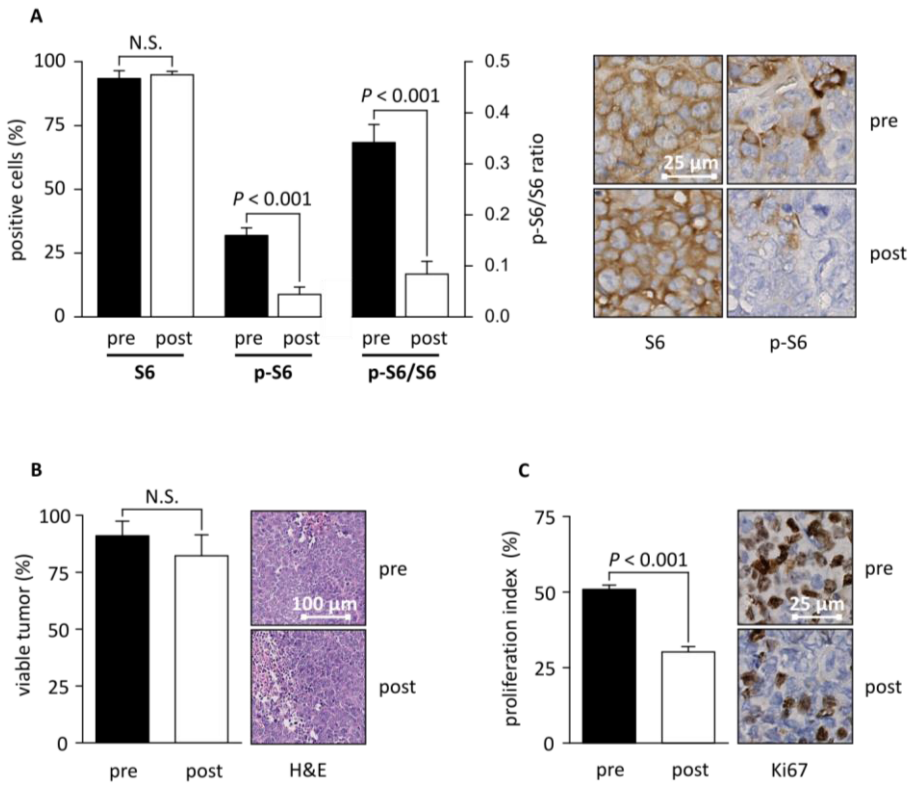


Figure 5. (A) S6 expression was homogeneously present in > 90% of tumor cells, while p-S6 was heterogeneously expressed. Everolimus treatment did not change total S6 expression, while p-S6 expression was significantly reduced in treated *versus* control tumors ($P < 0.001$). There was a marked decrease in the ratio between p-S6/S6, which was determined for each individual tumor. (B) Tumor viability is expressed as the percentage of viable tumor tissue within the total surface area analyzed. No significant differences in tumor viability were observed between treated and control tumors. (C) The proliferation index is expressed as the percentage of nuclei positive for Ki67. Compared to control tumors, everolimus treatment reduced the proliferating fraction by 41% ($P < 0.001$).

VEGF-A protein expression was lower in everolimus treated *versus* control tumors (0.33 ± 0.02 *versus* 0.50 ± 0.16 pg/µg total protein, $P = 0.04$) (**Figure 6A**). In addition, everolimus treatment reduced tumor vascularity, as the MVD in angiogenic hot-spots was 6.2 ± 1.7 in treated *versus* 10.6 ± 3.0 ($P < 0.001$) in control mice (**Figure 6B**).

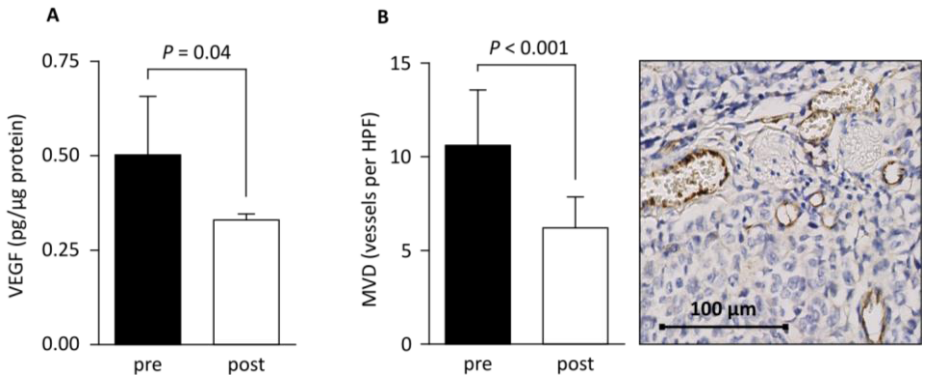


Figure 6. (A) VEGF-A levels determined in whole-tumor lysates show reduced VEGF-A in everolimus treated *versus* control tumors (P = 0.04). VEGF-A levels are expressed relative to the total protein content of each lysate, to correct for loading differences. (B) MVD, presented as the number of vWF positive vessels per angiogenic hot-spot, was reduced in treated *versus* control tumors (42% decrease, P < 0.001). A representative picture is included representing an angiogenic hot-spot in a control tumor.

DISCUSSION

This study shows that *in vivo* serial ^{89}Zr-bevacizumab PET imaging can visualize reduced tumor VEGF-A levels upon treatment with everolimus in an ovarian cancer xenograft model.

The relatively modest activity of temsirolimus observed in ovarian cancer patients underscores the need for patient selection. Responses limited to a subset of ovarian cancer patients have also been observed with VEGF(R)-targeted antiangiogenic drugs, as well as several other targeted agents, and could result from marked molecular heterogeneity which is characteristic to ovarian cancer.[24,25] The discovery of biomarkers for (early) response prediction might permit the selection of patients that are likely to benefit from single-agent mTOR inhibition or mTOR inhibitor-based combination regimens. Several proteins involved in mTOR signaling have been proposed as suitable biomarkers for response. Phosphorylation of mTOR in ovarian cancer specimens is a poor indicator of its kinase activity.[3] Phosphorylation of downstream target proteins (*e.g.* p70S6K, S6 and 4E-BP1) is a better read-out for mTOR activity and has been used to check for effective drug delivery and optimal biologic dosing in phase I clinical trials with everolimus.[18,26] Utilizing these protein expression levels as biomarkers for response is hampered by the need for (repeat) tumor tissue acquisition, or the use of surrogate tissues such as peripheral blood

mononuclear cells (PBMCs), skin or hair follicles. Instability of phosphorylated proteins further challenges the reliability (and reproducibility) of such analyses.[27]

In vivo molecular imaging provides an attractive alternative. It allows for whole-body, non-invasive monitoring of tumor biology and changes herein resulting from drug administration. These techniques are being explored as tools for patient selection for several molecular targeted drugs, including mTOR inhibitors. Thus far, ^{18}F-fluorodeoxyglucose PET (FDG-PET) measuring tumor glucose metabolism has received most attention.[28,29] Inhibition of mTOR does indeed reduce glucose uptake by reducing glucose transporter 1 (GLUT1) expression and/or hexokinase activity.[30] However, changes in FDG-PET according to EORTC criteria did not correlate with radiographic response according to RECIST criteria or progression-free survival in 34 patients with different cancer types treated with rapamycin.[28]

Given the role of mTOR in angiogenesis, we instead monitored the downstream effect of mTOR inhibition by everolimus on VEGF-A levels in xenografted ovarian cancers with ^{89}Zr-bevacizumab PET. Everolimus treatment resulted in reduced ^{89}Zr-bevacizumab tumor uptake, which coincided with delayed tumor growth, lower tumor VEGF-A protein expression and reduced MVD.

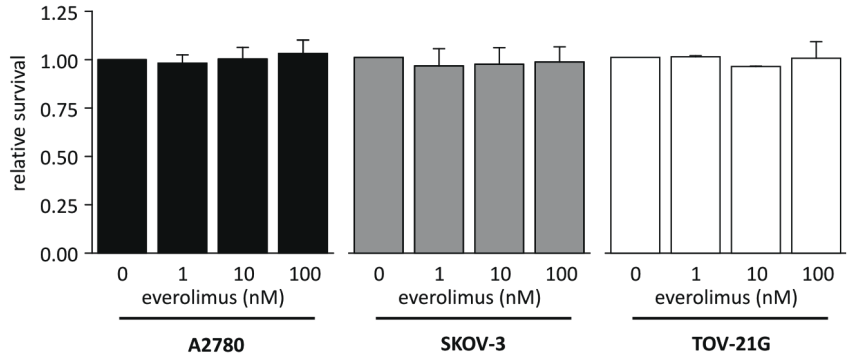
Accumulation of radiolabeled bevacizumab results from its interaction with larger human VEGF-A isoforms bound to (tumor) cell surfaces and extracellular matrix components.[31] For VEGF-A_{165}, the most abundant isoform, equilibrium is thought to exist between bound and freely diffusible fractions due to weak binding properties. ^{89}Zr-bevacizumab PET thus has the potential of not only visualizing presence but also activity of tumor-associated VEGF-A. This is supported by the finding that there was a 42% reduction in tumor vascularity (MVD) after everolimus treatment, demonstrating an antiangiogenic response. These findings are of interest to evaluate in the clinic. ^{89}Zr-bevacizumab PET is already known to show excellent tracer uptake in tumor lesions of renal cell cancer patients and is currently tested as an early biomarker for response to everolimus in 2 clinical trials (Everol*image*, NCT01028638; NETPET, NCT01338090).[32] Moreover, ^{89}Zr-bevacizumab PET might provide more insight in differential effects of antiangiogenic drugs on individual tumor lesions within a patient.

It is of interest that we observed a homogeneous decrease in tracer uptake after everolimus treatment, which was also observed previously with the heat shock protein 90 (HSP90) inhibitor NVP-AUY922.[14] This differs from previous findings with the VEGFR-targeted TKI sunitinib, which acts at the level of the endothelial cells. ^{89}Zr-ranibizumab, the radiolabeled Fab-fragment derived from bevacizumab, was used to

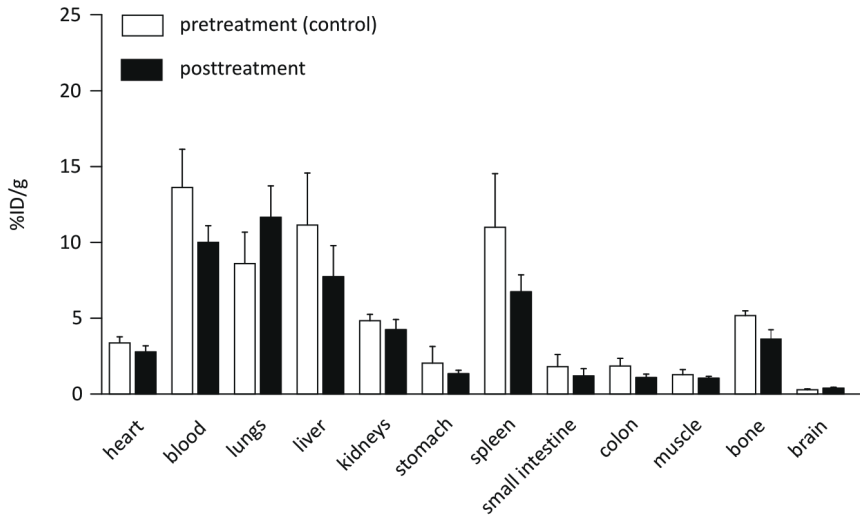
visualize the effect of sunitinib treatment on tumor VEGF-A levels in an ovarian cancer xenograft model.[15] Reduction of tracer uptake was more pronounced in the tumor center *versus* the rim of the tumor. Increased invasiveness was observed in xenograft models treated with sunitinib, and it was speculated that hypoxia (and consequently HIF activity) drives this adaptive response.[33] Lack of differential tracer uptake between tumor center and periphery observed with mTOR or HSP90 inhibitors might reflect their potential to inhibit HIF activity, through reducing its translation or stability.

Collectively, our data demonstrate that ^{89}Zr-bevacizumab PET provides a novel tool for non-invasive monitoring of antiangiogenic effects upon mTOR inhibitor therapy. It provides an interesting candidate biomarker in cancers in which angiogenesis inhibition is thought to underlie treatment efficacy, like ovarian cancer. Our results support the evaluation of ^{89}Zr-bevacizumab PET as an early biomarker for antiangiogenic efficacy of mTOR inhibitor therapy in clinical studies.

Supplementary data



Supplementary Figure S1. Fraction of viable ovarian cancer cells after 24 hours of everolimus exposure relative to untreated cells. Everolimus did not influence cell survival or proliferation in this treatment interval.



Supplementary Figure S2. Biodistribution data from non-tumor tissues collected from control animals after the baseline scans (pretreatment) and from treated animals after the second scanning sequence (posttreatment). Data expressed as percentage of the injected dose per gram of tissue (%ID/g).

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CHAPTER 5

DRUG-INDUCED CASPASE-8 UPREGULATION SENSITIZES CISPLATIN RESISTANT OVARIAN CARCINOMA CELLS TO RHTRAIL-INDUCED APOPTOSIS

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Br J Cancer **2011;104:1278-87**

ABSTRACT

BACKGROUND Drug resistance is a major problem in ovarian cancer. Triggering apoptosis using death ligands such as tumor necrosis factor-related apoptosis inducing ligand (TRAIL) might overcome chemoresistance.

METHODS We investigated whether acquired cisplatin resistance affects sensitivity to recombinant human (rh) TRAIL alone or in combination with cisplatin in an ovarian cancer cell line model consisting of A2780 and its cisplatin resistant subline CP70.

RESULTS Combining cisplatin and rhTRAIL strongly enhanced apoptosis in both cell lines. CP70 expressed less caspase-8 protein, while mRNA levels were similar compared with A2780. Pre-exposure of particularly CP70 to cisplatin resulted in strongly elevated caspase-8 protein and mRNA levels. Caspase-8 mRNA turnover and protein stability in the presence or absence of cisplatin did not differ between both cell lines. Cisplatin-induced caspase-8 protein levels were essential for the rhTRAIL-sensitizing effect as demonstrated using caspase-8 small-interfering RNA (siRNA) and caspase-8 overexpressing constructs. Cellular FLICE-inhibitory (c-FLIP) and p53 siRNA experiments showed that neither an altered caspase-8/c-FLIP ratio nor a p53-dependent increase in DR5 membrane expression following cisplatin were involved in rhTRAIL sensitization.

CONCLUSION Cisplatin enhances rhTRAIL-induced apoptosis in cisplatin resistant ovarian cancer cells, and induction of caspase-8 protein expression is the key factor of rhTRAIL sensitization.

INTRODUCTION

In ovarian cancer, the majority of tumors acquire drug resistance. Response rates to frontline platinum-based therapy are more than 80%, but most patients with advanced disease will finally relapse and die because of acquired drug resistance.[1] Chemoresistance is attributed to numerous mechanisms, which can be broadly divided into decreased DNA damage response via p53 and increased cell survival, mainly through defects in apoptosis.[2] A meta-analysis showed that aberrant p53 status results in a worse 5-year survival for ovarian cancer patients.[3]

Triggering apoptosis directly via the extrinsic apoptotic pathway might circumvent escape mechanisms developed by cancer cells. Especially the recombinant human (rh) form of the death ligand tumor necrosis factor-related apoptosis inducing ligand (TRAIL) is considered to be interesting for clinical use because of its ability to induce apoptosis in several types of cancer cell lines and xenografts.[4,5,6] Preliminary data from a phase I trial with rhTRAIL showed no major toxicity.[7]

TRAIL induces apoptosis by binding to death receptor 4 (DR4) and DR5.[6] Binding to these DRs causes receptor trimerization and recruitment of the adaptor protein Fas associated death domain (FADD). This in turn recruits caspase-8, resulting in the formation of the death inducing signaling complex (DISC).[8] Binding of caspase-8 to the DISC causes its activation[9], with subsequent activation of effector caspases-3, -6 and -7, which will execute apoptosis. The cellular FLICE-inhibitory protein (c-FLIP), which is vastly homologous to caspase-8 but lacks enzymatic activity, can also associate with the DISC, blocking activation of caspase-8 through competition for binding sites. It has also been stated that c-FLIP may function as an activator of caspase-8 under specific circumstances.[10,11]

In addition, the intrinsic apoptotic pathway can be activated by caspase-8 through cleavage of the BH3-only protein Bid that triggers perturbation of the mitochondria by Bax and Bak and finally activation of caspase-9 and effector caspases.[12] Numerous studies have demonstrated that drug resistance in cancer cells including ovarian cancer cells, could be prevented or overcome by combining rhTRAIL with chemotherapeutics.[13]

In a previous study, we showed that cisplatin can sensitize ovarian cancer cells to rhTRAIL-induced apoptosis *in vitro* as well as in a bioluminescent ovarian cancer xenograft model.[14] The described mechanisms involved in modulation in ovarian cancer cells were established by comparison of cell lines with different background and sensitivity patterns, which impede establishment of causal relationships. Therefore, we

investigated in an isogenic model of cisplatin resistance the molecular determinants for rhTRAIL sensitivity, the mechanism of synergy between cisplatin and rhTRAIL, and the role of functional p53 in this synergy.

METHODS

CELL LINES

The ovarian cancer cell lines A2780 and its 5-fold cisplatin resistant subline CP70, which carry wild-type and functional p53, were a kind gift from Dr Hamilton (Fox Chase Cancer Center).[15] Cisplatin resistance in CP70 can partly be explained by an increased DNA repair mechanism and a higher intracellular glutathione content.[16] Cells grew as monolayers in RPMI 1640 (Life Technologies, the Netherlands), supplemented with 10% heat-inactivated fetal calf serum (FCS) (Bodinco BV, the Netherlands) and 0.1 M L-glutamine. Cell lines were cultured in a humidified atmosphere with 5% CO_2.

CYTOTOXICITY ASSAY

The microculture tetrazolium assay was used to measure cytotoxicity as described earlier.[14] Treatment consisted of continuous incubation with cisplatin (Pharmachemie BV, the Netherlands) or rhTRAIL (produced as described earlier[17]) for 96 h. The mean IC50 ± standard deviation (SD) was determined in three experiments each performed in quadruplicate.

DETERMINATION OF APOPTOSIS

In 96-wells plates, cells were incubated with either rhTRAIL or cisplatin, or both. The cells were exposed to cisplatin for 4 h, washed with phosphate buffered saline (PBS: 6.4 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.14 mM NaCl, 2.7 mM KCl, pH = 7.2) twice and incubated in regular culture medium. At 20 h after administration of cisplatin, rhTRAIL was added for 4 h. Following rhTRAIL treatment, nuclear chromatin was stained with acridine orange to identify apoptosis by fluorescence microscopy.

SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS AND WESTERN BLOTTING

Cells were treated with rhTRAIL and/or cisplatin as described above. Treatment with the proteasome inhibitor MG132 (Calbiochem, the Netherlands) (0.5 μM) lasted for 24 h and rhTRAIL was added for the last 4 h. The caspase inhibitor I (zVAD) (Calbiochem) was added 2 h before MG132. Cycloheximide (CHX) (Sigma-Aldrich, the Netherlands) exposure was performed as indicated. After treatment, total cell lysates were

generated and Western blotting was performed as described previously[14], using the following primary antibodies: Fas-associated death domain (FADD) and XIAP (Transduction Laboratories, the Netherlands), Bax, Bcl-2, Bcl-XL and p53-DO-1 (Santa Cruz, the Netherlands), caspase-9 and Cip1/Waf1 (p21) (BD Biosciences, the Netherlands), caspase-8 and cleaved caspase-3 (Cell Signaling, the Netherlands). The Bid and FLIP NF6 antibodies were kindly provided by Dr J Borst (Netherlands Cancer Institute) and Dr ME Peter (Ben May Institute for Cancer Research), respectively. Secondary antibodies conjugated with horseradish peroxidase were obtained from DAKO (Denmark). Equal protein loading was confirmed by β-actin (ICN Pharmaceuticals, the Netherlands). Visualization was performed with BM chemiluminescence blotting substrate (POD) or Lumi-Light^{plus} Western blotting substrate (Roche Diagnostics, the Netherlands).

FLOW CYTOMETRY

Cells were collected using trypsin and washed once with cold PBS. Cells were subsequently washed twice with cold PBS containing 2% FCS and 0.1% sodium azide and incubated with phycoerythrin (PE)-conjugated mouse monoclonal antibodies against DR4, DR5, decoy receptor 1 (DcR1) and DcR2. Mouse PE-labelled IgG1 and IgG2B were used as isotype controls. All PE-labeled antibodies were purchased from R&D Systems (UK). Receptor membrane expression was analyzed using a flow cytometer (Epics Elite, Coulter Electronics, USA) and is shown as mean fluorescent intensity (MFI) of all analyzed cells.

RNA INTERFERENCE AND GENE TRANSFECTION

Small-interfering RNAs (siRNAs) were synthesized by Eurogentec (Belgium). The double-stranded c-FLIP siRNA was 5'GAGGUAAGCUGUCUGUCGG-dTdT3' (sense) and 5'CCGACAGACAGCUUACCUC-dTdT3' (antisense). P53 siRNA 5'GCAUGAACCGGAGGCCCAU-dTdT3' (sense) and 5'AUGGGCCUCCGGUUCAUGC-dTdT3' (antisense) was designed according to Martinez *et al*.[18] Oligonucleotides specific for luciferase mRNA served as a negative control.[19] Caspase-8 siRNA 5'CUACCAGAAAGGUAUACCU-dTdT3' (sense) and 5'AGGUAUACCUUUCUGGUAG-dTdT3' (anti-sense) was designed according to Chun *et al*[20] and negative control siRNA from Eurogentec was used. Subconfluent cells were incubated in unsupplemented Optimem medium and transfected with siRNA (up to 133 nM) using Oligofectamine reagent according to the manufacturer's protocol (Invitrogen, the Netherlands). For

the caspase-8 overexpression experiments, cells were transfected with pcDNA3-FLICE (a gift from J Borst) using Fugene. The next day, cells were treated with cisplatin and/or rhTRAIL as described above, collected and used for protein isolation, cytospins, FACS experiments, and/or an apoptosis assay. Cytospins were immunohistochemically stained for caspase-8 as described earlier.[21]

REAL-TIME RT-PCR

Total RNA was isolated by guanidine isothiocyanate-phenol-chloroform extraction with TRIzol (Invitrogen) and purified using the RNeasy mini Kit and on-column DNase I digestion (Qiagen, the Netherlands) according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized from 1600 ng purified RNA as described by the manufacturer's protocol (Life Technologies) using oligo (dT)11 primers and MMLV transcriptase. Real-time RT-PCR was performed in 96-wells plates on a MyiQ real-time detection system (BioRad, the Netherlands) with GAPDH as housekeeping reference gene. Primer sequences of caspase-8 were 5'-GGAGCTGCTCTTCCGAATTA-3' (forward) and 5'-GCAGGTTCATGTCATCATCC-3' (reverse) and those of GAPDH were 5'-CACCACCATGGAGAAGGCTGG-3' (forward) and 5'-CCAAAGTTGTCATGGATGACC-3' (reverse). Amplification of the samples in triplicate was carried out in a final reaction volume of 25 µL, containing 1x iQ SYBR Green Supermix (BioRad), 5 µM of each primer and 5 µL cDNA (1:50). The thermocycling programme used for each real-time RT-PCR consisted of an initial 3 min denaturation at 95°C, followed by 40 cycles of 15 s denaturation at 95°C, 20 s primer annealing at the primer specific T_{ann} and 30 s fragment elongation at 72°C. A melting curve was obtained at the end of each 40 cycles of amplification in order to determine the presence of a unique reaction product. To determine RT-PCR efficiency and initial starting quantity of the samples, a standard curve was generated using samples from a 1:3 dilution series of total starting cDNA.

STATISTICAL ANALYSIS

All experiments were performed at least three times on different occasions. Analysis included double-sided, unpaired Student's *t*-test. A *P*-value < 0.05 was considered significant.

RESULTS

COMBINATION OF CISPLATIN AND RHTRAIL CAUSES ENHANCED INDUCTION OF APOPTOSIS

A2780 was moderately sensitive to cisplatin treatment for 96 h, with an IC50 of 2.6 µM in a survival assay. CP70 was 5-fold resistant to cisplatin, with an IC50 of 14.7 µM (**Figure 1A**). The subline CP70 was rhTRAIL resistant, and A2780 was moderately sensitive, as IC50 was not reached using up to 0.25 µg/ml in a survival assay for 96 h (**Figure 1B**). On the basis of previous data, cells were treated for 4 h with cisplatin, recovered for 20 h and treated with rhTRAIL for 4 h for the apoptosis assay.[14] rhTRAIL (0.25 µg/ml) induced moderate levels of apoptosis in A2780, ~20%, whereas CP70 was not sensitive to rhTRAIL. Combination of cisplatin with rhTRAIL enhanced apoptosis in both cell lines, with ~80% apoptosis in A2780 and ~40% apoptosis in CP70 (**Figure 1C**).

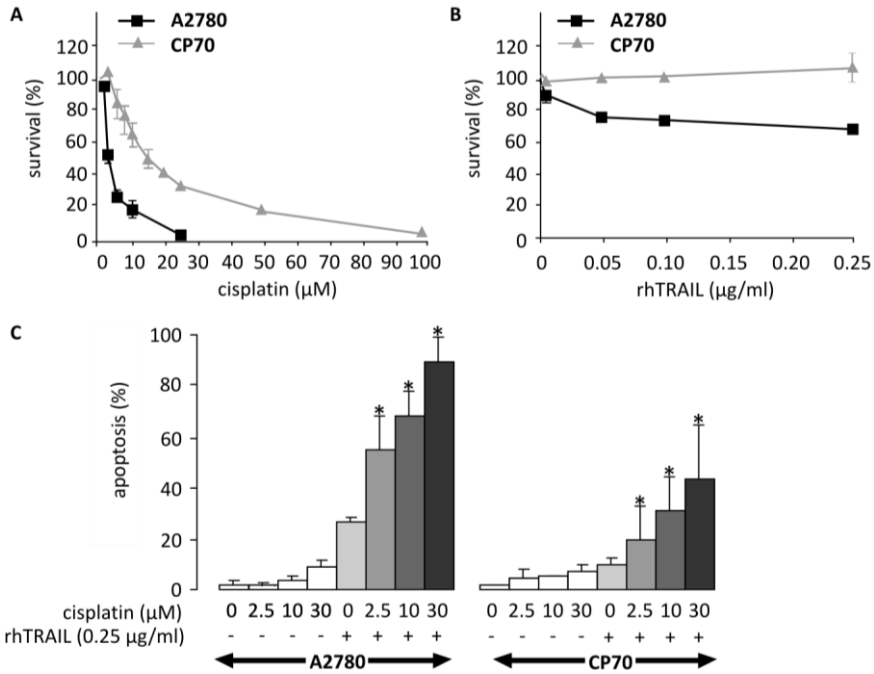


Figure 1. Resistance to cisplatin causes cross-resistance to rhTRAIL. Combination therapy overcomes resistance. (A) Survival after 96 h exposure to 0-25 µM (A2780) or 0-100 µM (CP70) cisplatin and (B) survival after 96 h exposure to 0-0.25 µg/ml rhTRAIL as measured by cytotoxicity assays. (C) To determine apoptosis induction, cells were treated for 4 h with cisplatin (2.5, 10 and 30 µM) after which cisplatin was washed away. Twenty hours later, the cells were treated for 4 h with 0.25 µg/ml rhTRAIL. Apoptosis was determined with acridine orange staining. Apoptosis in the combinations , marked with *, was significantly enhanced ($P < 0.05$) over apoptosis after single-agent treatment. Data represent the mean ± SD of at least three independent experiments.

THE RESISTANT CELL LINE CP70 HAS REDUCED CASPASE-8 PROTEIN LEVELS

To determine which cellular characteristics could account for the different sensitivity patterns to rhTRAIL, key components of the TRAIL signaling pathway were analyzed. Membrane expression of DR4 and DcR1 were almost undetectable in both cell lines. DcR2 expression was similar, whereas DR5 was increased in CP70 (MFI = 120 ± 31) *versus* A2780 (MFI = 53 ± 26) (**Figure 2A**). Western blot analysis showed similar expression of FADD, c-FLIP, caspases-9 and -3, Bid, Bax, Bcl-2, Bcl-XL and XIAP levels in A2780 and CP70. Remarkably, caspase-8 levels were lower in CP70 than in A2780 (**Figure 2B**).

CISPLATIN INDUCES UPREGULATION OF CASPASE-8 PROTEIN

Next, we evaluated the effect of cisplatin on TRAIL receptor expression and the effect of different regimens on cleavage of caspases-8 and -9. Cisplatin did not affect DR4 and DcR1 levels. DcR2 was moderately upregulated, but DR5 was strongly induced upon exposure to cisplatin in both cell lines (**Figure 2C**). In response to cisplatin, moderate activation of caspase-8 was induced in A2780. rhTRAIL alone induced mild activation of caspases-8 and -9, whereas upon combination treatment, both caspases were strongly activated. In CP70, cisplatin induced upregulation of procaspase-8 levels. rhTRAIL induced a slight activation of caspases-8 and -9, while the combination of cisplatin and rhTRAIL resulted in strong activation of both caspases (**Figure 2D**).

CASPASE-8 PROTEIN LEVELS AFFECT RHTRAIL-INDUCED APOPTOSIS

As caspase-8 protein levels are reduced in CP70 compared with A2780, we examined the importance of caspase-8 protein levels for rhTRAIL sensitivity following cisplatin pretreatment. Efficient downregulation of caspase-8 with siRNA strongly reduced apoptosis induction following treatment of A2780 and CP70 with rhTRAIL alone and combined with cisplatin (**Figure 3A**). In caspase-8 siRNA-transfected cells, combined treatment with cisplatin and rhTRAIL minimally induced activation of caspases-8 and -3, whereas in negative control siRNA-transfected cells strong activation of both caspase-8 and caspase-3 was observed (**Figure 3B**). It can be noticed that caspase-3 levels are also slightly upregulated following cisplatin in both cell lines (see **Figure 3B**). A role for caspase-3 in rhTRAIL sensitization can therefore not be excluded. However, the importance of caspase-8 levels was also demonstrated by transient upregulation of caspase-8 in CP70 that enhanced apoptosis induction by rhTRAIL alone and by the rhTRAIL *plus* cisplatin combination (**Figure 3C** and **D**).

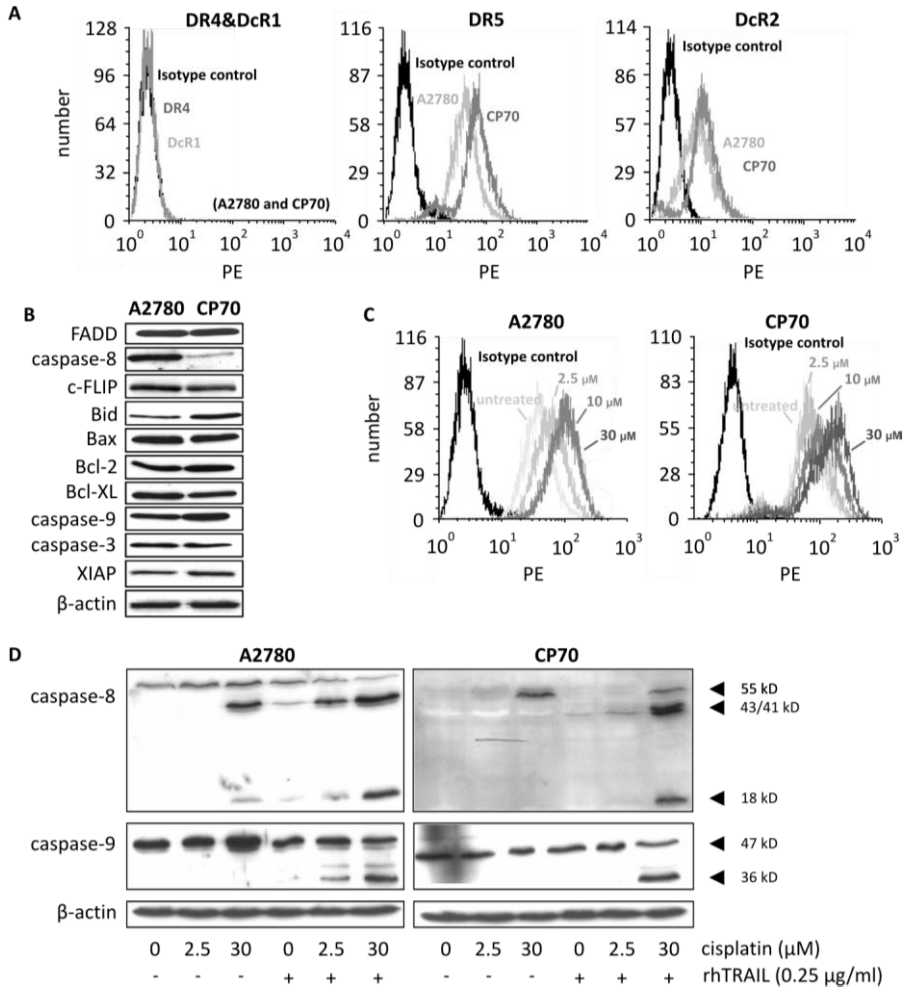


Figure 2. Cellular characteristics involved in sensitivity to rhTRAIL. Cisplatin can induce caspase-8 protein in the low caspase-8 expressing CP70. (A) Basic levels of TRAIL receptor membrane expression in A2780 and CP70 as determined by FACS analysis. Receptor expression is expressed as mean fluorescence intensity (MFI). (B) Western blot analysis of basic protein expression levels of key determinants of the TRAIL pathway in A2780 and CP70. (C) The effect of cisplatin on DR5 expression in both cell lines. Flow cytometry was performed at 24 h after 4 h incubation with cisplatin. (D) Western blot analysis of caspase-8 and caspase-9 after exposure to cisplatin and rhTRAIL. A2780 and CP70 were incubated with 2.5 or 30 μM cisplatin during 4 h, after which the cells were washed. Twenty hours later, unexposed or cisplatin-exposed cells were treated during 4 h with 0.25 μg/ml rhTRAIL. β-actin serves as a loading control. The blots are representative for at least three independent experiments.

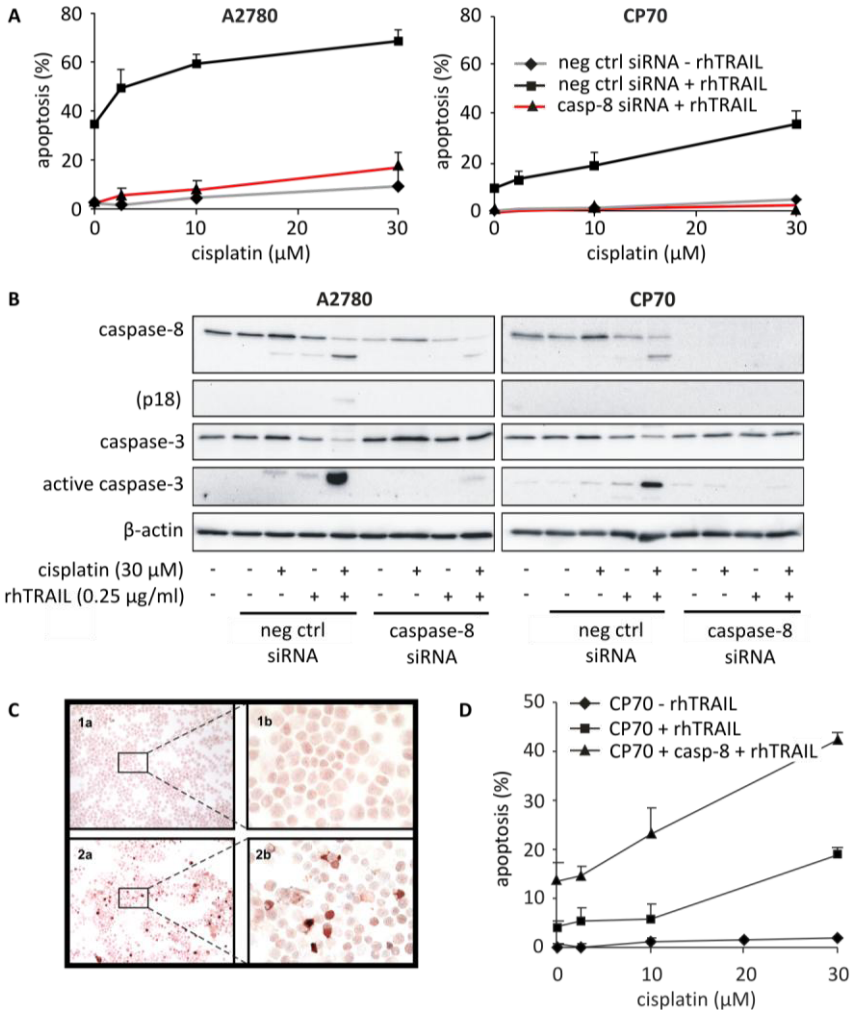


Figure 3. Caspase-8 downregulation inhibits cisplatin- and rhTRAIL-induced apoptosis, whereas caspase-8 upregulation augmented apoptosis induction. A2780 and CP70 were transfected with siRNA against caspase-8 or negative control siRNA (A and B). The CP70 cells were transiently transfected with a caspase-8 construct (C and D). At 48 h after transfection, cells were treated with 30 μM cisplatin or medium control for 4 h, after which all cells were washed with PBS. Following 16 h of recovery, cells were treated with 0.25 μg/ml rhTRAIL or medium control for 4 h. (A) Apoptosis induction was determined by acridine orange staining. Caspase-8 siRNA strongly reduced apoptosis induction by rhTRAIL and cisplatin in both cell lines. (B) Caspase-8 and caspase-3 expression was determined using Western blot analysis. The exposure time of the caspase-8 blot of CP70 was increased compared to the blot of A2780. β-actin serves as a loading control. The blots are representative for at least three independent experiments. (C) Cytospins of CP70 cells were generated and stained for caspase-8 and show increased caspase-8 levels following transfection (2a + b) compared with untransfected cells (1a + b). (D) Apoptosis induction following cisplatin and rhTRAIL was determined by acridine orange staining. Caspase-8 upregulation increased apoptosis induction by rhTRAIL alone and by rhTRAIL in combination with cisplatin.

CISPLATIN INCREASES CASPASE-8 MRNA LEVELS

We investigated the mechanism causing reduced basal caspase-8 protein levels and cisplatin-induced caspase-8 upregulation in CP70. Basal caspase-8 mRNA levels were slightly higher in CP70 than in A2780. Exposure to cisplatin resulted in 1.5-fold induction of caspase-8 mRNA in both cell lines (**Figure 4A**). Possible differences in mRNA stability over time and in response to cisplatin were determined using actinomycin D. Caspase-8 mRNA degradation in the absence or presence of cisplatin was not different between A2780 and CP70 (P = 0.35) (**Figure 4B**). These results show that cisplatin does not influence caspase-8 mRNA degradation, indicating that the induction of mRNA is caused by increased transcription.

CASPASE-8 PROTEIN TRANSLATION IS REDUCED IN CP70

The similar caspase-8 mRNA expression, whereas different basal caspase-8 protein levels were observed, suggest possible changes in caspase-8 protein translation or degradation in CP70. Cells were exposed to the proteasome inhibitor MG132 to test whether increased proteasomal degradation causes the reduced caspase-8 protein levels in CP70. Treatment with MG132 induced upregulation of active caspase-8, leading to concomitant synergy with rhTRAIL (**Figure 4C**). To prevent caspase-8 activation by MG132 treatment, cells were co-incubated with the caspase inhibitor zVAD for 24 h, which did not lead to a change in full-length caspase-8 levels (**Figure 4D**). Cycloheximide (CHX) exposure for up to 24 h slightly affected caspase-8 levels in both cell lines, whereas caspase-8 degradation was not different between A2780 and CP70 (**Figure 4E**). These results show that not caspase-8 protein stability but rather a decreased translation of caspase-8 mRNA is causing the reduced caspase-8 expression in CP70.

INCREASED CASPASE-8 BUT NOT THE CASPASE-8/C-FLIP RATIO IS INVOLVED IN THE RESPONSE TO CISPLATIN AND RHTRAIL IN CP70

Difference in caspase-8/c-FLIP ratio between A2780 and CP70 might contribute to resistance in CP70. However, efficient downregulation of both c-$FLIP_L$ and c-$FLIP_S$ with siRNA did not lead to increased apoptosis after either treatment regimen, and did not affect caspase-8 cleavage (**Figure 5A**). Downregulation even led to a significant decrease in apoptosis in CP70 (**Figure 5B**). These results indicate that solely the elevated caspase-8 level is involved in the onset of apoptosis after combination treatment in CP70. Moreover, c-FLIP even promotes caspase-8 activation in these cells.

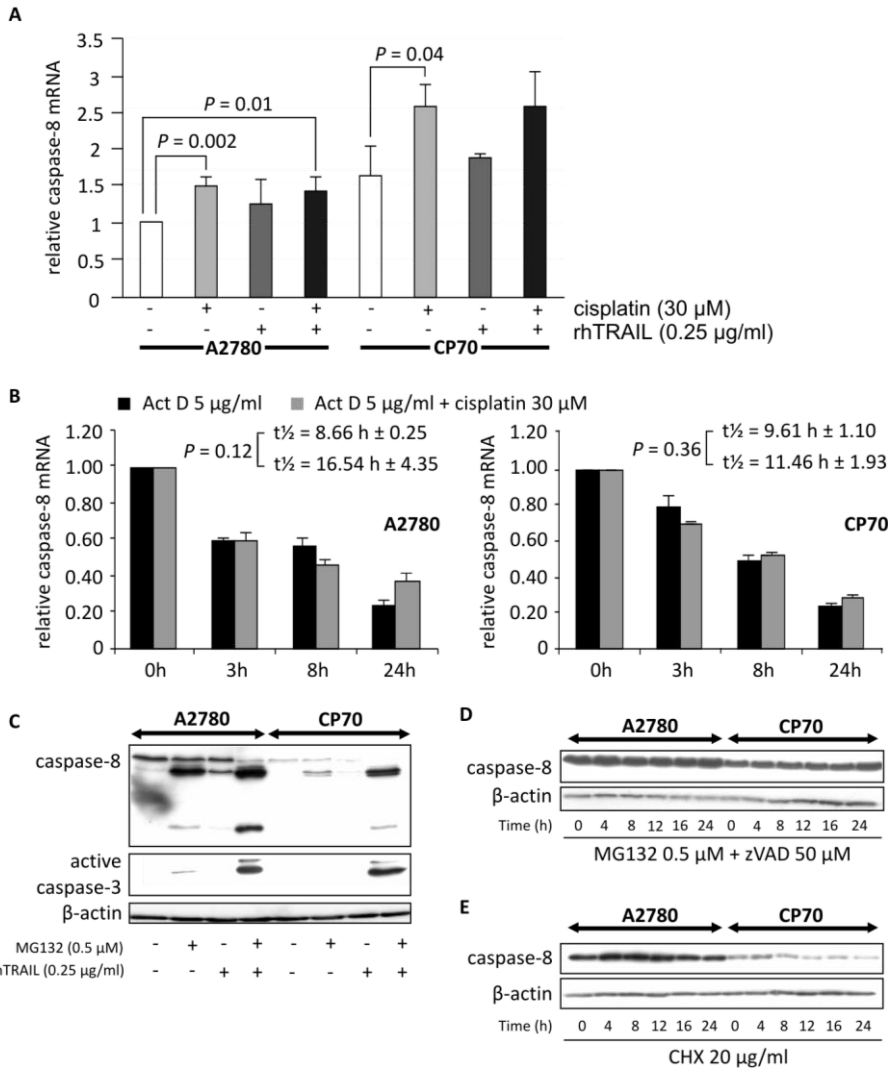


Figure 4. Stability of caspase-8 mRNA or protein does not differ between A2780 and CP70. Differences in caspase-8 protein levels in CP70 are due to changes in protein translation. (A) Expression of caspase-8 mRNA was determined with quantitative RT-PCR after exposure to 30 µM cisplatin, 0.25 µg/ml rhTRAIL or the combination in both cell lines. Cells were incubated for 4 h with cisplatin, after which the cells were washed and total RNA was isolated 20 h after cisplatin exposure. Total RNA was isolated 4 h after exposure to rhTRAIL. Quantitative RT-PCR was carried out on cDNA with the SYBR Green method. Quantification was performed with the standard curve method with GAPDH as reference. Basic mRNA levels between A2780 and CP70 were not different. Cisplatin induced caspase-8 mRNA by 1.5-fold. (B) To assess caspase-8 mRNA stability, cells were exposed to 30 µM cisplatin for 4 h or left untreated. Hereafter, all conditions were washed with PBS and received new medium, followed by addition of 5 µg/ml actinomycin D (Act D). Total RNA was extracted at the time of Act D addition (t = 0 h) and at the indicated timepoints. Quantitative RT-

PCR was performed as in (A). Data represent the mean ± SD of at least three independent experiments. (C) A2780 and CP70 were exposed to 0.5 μM of the proteasome inhibitor MG132 for 24 h. The following day either rhTRAIL was added the last 4 h of incubation, or the cells were left untreated. Then cells were lysed and subjected to Western blot analysis with caspase-8 and caspase-3 antibodies. (D and E) The A2780 and CP70 cell lines were treated with 0.5 μM MG132 for the indicated timepoints in the presence of 50 μM of zVAD and lysed or treated with 20 μg/ml cycloheximide (CHX) for the indicated timepoints and lysed. Following SDS-PAGE, immunoblotting was carried out with caspase-8 antibodies. β-actin serves as a loading control. All immunoblots are representative for at least three independent experiments.

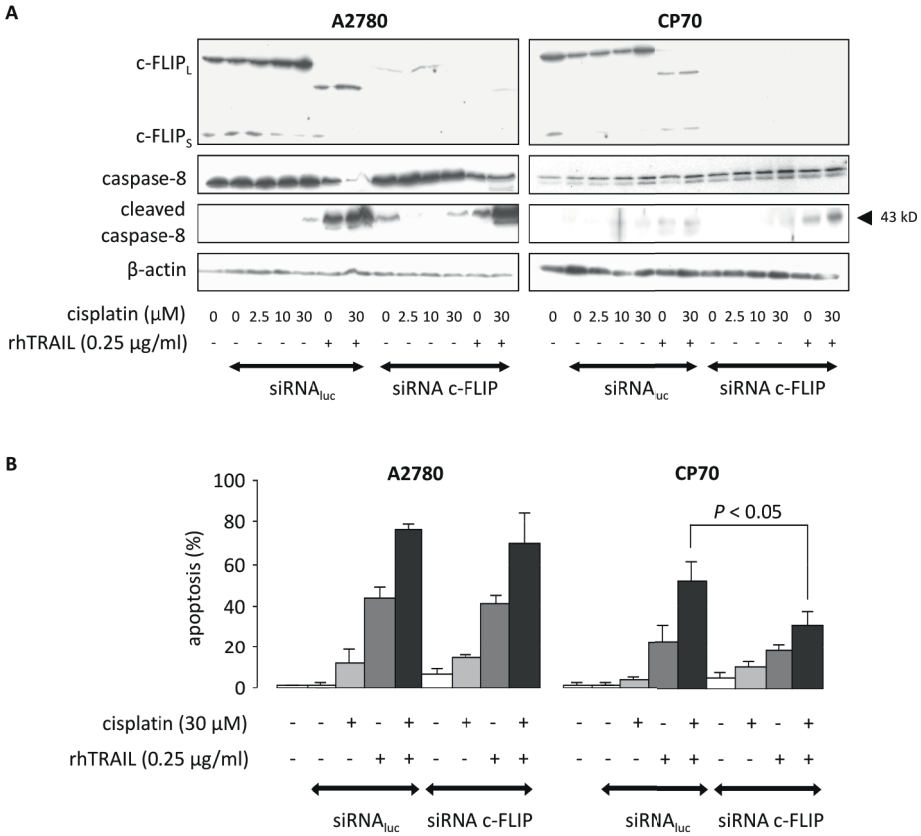


Figure 5. The caspase-8/c-FLIP ratio is not involved in resistance to rhTRAIL. Cells were transfected with siRNA against c-FLIP or luciferase. The next day, cells were exposed to cisplatin for 4 h, after which all cells including those conditions left unexposed were washed. The following day after 4 h incubation with or without 0.25 μg/ml rhTRAIL, cell lysates were made. (A) c-FLIP and caspase-8 cleavage was determined with Western blot analysis. The exposure time of the caspase-8 blot of CP70 was increased compared to the blot of A2780. The blots are representative for at least three independent experiments. (B) A small fraction of the same cell suspension used for Western blot was plated in a 96-wells plate and apoptosis levels were determined with acridine orange apoptosis assays. Data represent the mean ± SD of at least three independent experiments. After siRNA against c-FLIP, a significant decrease in apoptosis occurred in CP70 after treatment with cisplatin and rhTRAIL ($P < 0.05$).

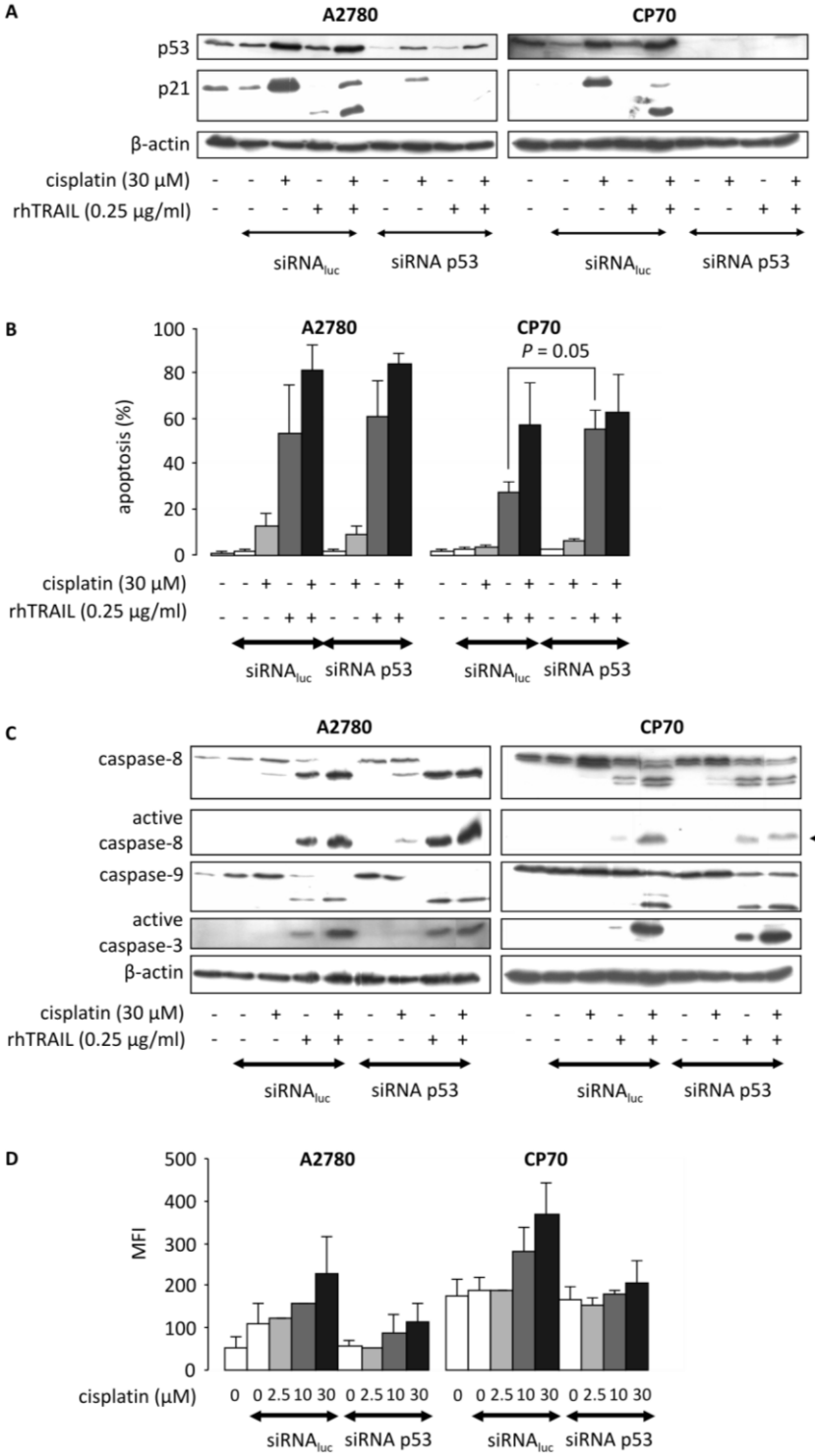
P53 CAUSES CISPLATIN-INDUCED DR5 EXPRESSION BUT IS NOT INVOLVED IN RHTRAIL SENSITIZATION

As p53-induced upregulation of DR5 is frequently described to be instrumental in the synergistic effect between chemotherapeutics and rhTRAIL[22], we asked whether the synergistic effect of cisplatin and rhTRAIL was p53-dependent. The tumor suppressor protein p53 was efficiently and functionally downregulated using siRNA in A2780 and CP70 as shown by the decreased expression of p21 (Waf1/Cip1), a transcriptional target of p53 (**Figure 6A**). In response to cisplatin, p53 levels rose slightly in p53-suppressed cells, but the levels remained far below those in the untreated luciferase siRNA transfected cells. Apoptosis induction (**Figure 6B**) and activation of caspases-8, -9 and -3 (**Figure 6C**) were not affected by p53 siRNA. Following p53 downregulation, basal DR5 membrane expression maintained unchanged, whereas cisplatin-induced DR5 membrane expression was effectively suppressed in A2780 and CP70 (**Figure 6D**). These results show that p53-dependent upregulation of DR5 is not involved in the synergy between cisplatin and rhTRAIL. In addition, DISC-IP using a DR5 antibody following cisplatin treatment showed that DISC-formation is not impeded in CP70 (data not shown).

Figure 6 (next page). The tumor suppressor protein p53 causes cisplatin-induced DR5 expression but is not required for rhTRAIL sensitization of A2780 and CP70. A2780 and CP70 cells were transfected with siRNA against p53 or luciferase and 24 h later exposed to 30 μM of cisplatin during 4 h, after which all cells including those not exposed to cisplatin, were washed. The next day the cells were treated with 0.25 μg/ml rhTRAIL for 4 h. (A) Subsequently, Western blotting of p53 and p21 levels was performed on the lysates. β-actin serves as a loading control. (B) A small fraction of the same cell suspension used for Western blot was plated in a 96-wells plate and apoptosis levels were determined with acridine orange apoptosis assays. After downregulation of p53, a significant increase in apoptosis occurred in CP70 after treatment with rhTRAIL. (C) Cleavage patterns of caspase-8, caspase-9 and caspase-3 were determined on lysates after transfection with siRNA against luciferase and p53 as indicated above. The exposure time of the caspase-8 blot of CP70 was increased compared to the blot of A2780. β-actin serves as a loading control. All blots are representative for at least three independent experiments. (D) At 24 h after siRNA transfection, the cells were exposed for 4 h to 2.5, 10 and 30 μM cisplatin, after which all cells were washed. The next day cells were collected and analysed for DR5 expression by flow cytometry. DR5 expression is represented as MFI. Data represent the mean ± SD of at least three independent experiments.

DISCUSSION

In the present study, we show that the cellular caspase-8 protein level is an important determinant of sensitivity to rhTRAIL-induced apoptosis in an isogenic ovarian cancer cell line model of acquired cisplatin resistance. Combination of cisplatin and rhTRAIL


A
A2780
CP70
p53
p21
β-actin
cisplatin (30 μM)
rhTRAIL (0.25 μg/ml)
siRNA$_{luc}$
siRNA p53
B
apoptosis (%)
P = 0.05
C
caspase-8
active caspase-8
18 kD
caspase-9
active caspase-3
D
MFI
0 0 2.5 10 30

effectively induced apoptosis, with cisplatin-induced caspase-8 protein expression being the key factor of sensitization to rhTRAIL in CP70. The central role of caspase-8 was further indicated by our observation that cisplatin-induced DR5 upregulation was not an important sensitizing factor. As caspase-8 mRNA levels do not differ significantly between A2780 and CP70, the low caspase-8 protein levels in CP70 are likely to be caused by decreased translation of caspase-8 protein rather than decreased transcription through methylation or genetic alterations that occur in neuroblastoma[23,24] and in other solid tumors.[25,26] Increased caspase-8 protein degradation in CP70 was ruled out, as the effect of inhibition of protein synthesis and proteasomal degradation on caspase-8 levels was not different between A2780 and CP70.

Translational and posttranslational modifications are important factors of the expression levels and activity of key proteins in the regulation of cell survival and apoptosis such as p53[27], p73[28], XIAP and APAF-1.[29] Recently, attention has been drawn to microRNAs (miRNAs) as important regulators of translation and mRNA stability.[30] Deregulation of several miRNAs was described for ovarian cancer, and an important role for miRNAs in cisplatin and TRAIL resistance was shown in ovarian and lung cancer, respectively.[31,32] In melanoma cells, an inducible post-translational modification of mRNA contributed to TRAIL resistance, in which cytosolic proteins could suppress DR5 protein expression by binding to the 3'-untranslated region of DR5 mRNA.[33] These studies support our hypothesis that a posttranscriptional mechanism is involved in the low expression of caspase-8 in CP70. The fact that, most prominently in CP70, caspase-8 translation could be enhanced by cisplatin exposure argues in favor of a reversible block of translation. Low caspase-8 levels were previously shown to contribute to rhTRAIL resistance in several cell line models and tumors.[34,35] Regarding the role of caspase-8 and the extrinsic apoptotic pathway in chemotherapy-induced cell death, conflicting reports have been published. Death receptor-dependent and -independent activation of caspase-8 was involved in the response to different chemotherapeutic drugs.[36,37,38] In addition, resistance to these drugs through defects in the extrinsic pathway further supported the involvement of this pathway in chemotherapy-induced cell death.[37,39]

Besides the differential expression of caspase-8, we found no other proteins that could account for the difference in TRAIL sensitivity between A2780 and CP70. As caspase-3 was slightly upregulated in both cell lines following cisplatin, a role for caspase-3 and hence also for XIAP cannot be completely ruled out.[40] However, basal

caspase-3 levels did not differ between the two cell lines, excluding caspase-3 as a determinant for the difference in rhTRAIL sensitivity. A reduced caspase-8/c-FLIP ratio can result in resistance to death receptor signaling[41,42], whereas an increased caspase-8/c-FLIP ratio in the DISC can cause sensitization to rhTRAIL by chemotherapeutics.[43,44] In our cell lines, downregulation of c-FLIP did not increase apoptosis induction. In the resistant CP70, downregulation of c-FLIP even resulted in a significant drop in apoptosis levels after exposure to cisplatin and rhTRAIL. This suggests that c-FLIP functions as a proapoptotic protein in CP70 and that the caspase-8 level is the most important determinant for rhTRAIL sensitivity. Although extensive literature exists on the anti-apoptotic function of c-FLIP, a role of c-FLIP in activation of caspase-8 was also reported.[10,11] It is postulated that the c-FLIP$_L$ concentration at the DISC determines whether caspase-8 activation or inhibition occurs.[10,45] Thus, it can be reasoned that the caspase-8-activating function of FLIP applies to A2780 and CP70, especially when low caspase-8 levels are present and heterodimerization can occur more easily than homodimerization.

Upregulation of DR4 or DR5, which can occur in a p53-dependent or independent manner in response to chemotherapeutics[46,47], is often described as a key event in the synergistic effect between chemotherapeutics and rhTRAIL.[22] In this study, we show that the increase of DRs after chemotherapy might just be an epiphenomenon, instead of a key event in modulation of rhTRAIL-induced cell death by chemotherapeutics. Downregulation of p53 did not affect apoptosis induction in both cell lines, whereas the substantial increase of DR5 after treatment with cisplatin was almost completely abrogated by p53 downregulation. It has been shown that p53 is required for cisplatin-induced apoptosis either via p53-dependent ubiquitination of FLIP- or p53-induced XIAP and Akt downregulation when used as a single-agent.[40,48] These results may explain why CP70 has reduced caspase-8 levels compared with A2780. However, in our experiments we have chosen to use cisplatin in a short treatment setting. This was followed by a short treatment with rhTRAIL, and we have demonstrated that upon combination of cisplatin and rhTRAIL, p53 is no longer required for apoptosis induction.

Conclusively, these results show that cisplatin enhances rhTRAIL sensitivity in both cisplatin sensitive and resistant cells. Induction of caspase-8 protein expression is the key driver of rhTRAIL sensitization.

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CHAPTER 6

APO010, A FAS LIGAND MULTIMER, IS AN EFFECTIVE INDUCER OF APOPTOSIS IN CHEMOTHERAPY SENSITIVE AND RESISTANT HUMAN SOLID CANCER CELL LINES

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Submitted for publication

ABSTRACT

BACKGROUND Targeting cancer cells by activating proapoptotic members of the tumor necrosis factor (TNF) receptor family may circumvent chemoresistance. MegaFasL (APO010) is a hexameric form of soluble Fas ligand (FasL), which can activate the extrinsic apoptosis pathway by binding its cognate receptor Fas. Aim of this study was to determine the apoptosis-inducing potential of APO010 in human solid cancer cell lines with various resistance profiles, and its ability to circumvent resistance to chemotherapy and the death ligand TNF-related apoptosis inducing ligand (TRAIL).

METHODS Cell survival, apoptosis induction, caspase-3 activation, Fas membrane expression and the expression of death inducing signaling complex components was determined in a panel of 10 human solid cancer cell lines. These cell lines comprised 3 isogenic resistance models.

RESULTS Several cell lines showed reduced cell survival and increased apoptosis induction by APO010. Sensitivity to APO010 was independent of inherent resistance to chemotherapy and partially related to TRAIL resistance. Fas membrane expression, determined by flow cytometry, correlated qualitatively with APO010 sensitivity. Pretreatment with cisplatin induced upregulation of Fas membrane expression and rendered cells sensitive to APO010 independently of inherent cisplatin resistance or initial insensitivity to APO010 monotherapy.

CONCLUSION APO010 is a potent inducer of apoptosis in a number of drug sensitive and resistant solid cancer cell lines. No consistent cross-resistance was observed between APO010 and conventional chemotherapeutic drugs, indicating APO010 can be used to circumvent chemoresistance. In addition, combining APO010 with platinum-based chemotherapy can enhance its antitumor efficacy.

INTRODUCTION

Inherent or acquired resistance to chemotherapy is a major cause for treatment failure in patients with solid malignancies. Chemotherapy resistance is often not specific for one class of chemotherapeutic agents and cross-resistance to a number of unrelated agents can occur. Targeting cancer cells by activating proapoptotic members of the tumor necrosis factor (TNF) receptor family may provide a potential cellular pathway that can be used to circumvent chemoresistance. Recombinant human TNF-related apoptosis inducing ligand (rhTRAIL) as well as agonistic TRAIL-receptor antibodies have been applied to *in vitro* models and showed activity against ~50% of the cell lines. Moreover, these drugs have already entered the clinic, are well tolerated in patients, but response rates to single-agent treatment are low.[1]

Fas ligand (FasL, CD95L) signals cell death by activation of its cognate receptor Fas (CD95), which is another member of the TNF receptor family. Upon binding by FasL, Fas is trimerized and its intracellular death domain interacts with the adaptor protein Fas-associated death domain (FADD).[2] FADD recruits procaspases-8 and -10 and the death inducing signaling complex (DISC) is formed. Subsequent activation of the initiator caspases (caspases-8 and -10) by autocleavage induces a cascade of effector caspases, ultimately resulting in apoptosis.

Various cancer cell lines, as well as short-term cultures of patient-derived cancer cells, express Fas on their membranes. A number of these cell lines are shown to be sensitive to agonistic anti-Fas antibodies or recombinant soluble FasL (sFasL).[3] Although anti-Fas antibody treatment appeared to be extremely hepatotoxic in mice, probably due to high Fas membrane expression found on the murine hepatocytes, treatment with sFasL showed less toxicity.[4] FasL is mostly membrane-bound under physiologic conditions, but can be processed to a soluble form and shed by metalloproteases. This sFasL is less effective in inducing apoptosis compared to its membrane-bound form[5,6], but cross-linking sFasL restores the proapoptotic properties of FasL.[5] A sFasL hexamer, consisting of two trimers held in close proximity, is the minimal ligand structure required to induce apoptosis to a similar extent as membrane-bound FasL.[7] Therefore, two sFasL trimers have been linked to one another with the C-terminal collagen domain of adiponectin (ACRP30), to which the conservative TNF family domain of FasL has structural homology.[8] This MegaFasL (APO010) provides an interesting therapeutic approach to exploit the potential of Fas-mediated apoptosis induction.[9] Currently, it is under phase I clinical investigation [NCT00437736].

In addition, cancer cell lines can be sensitized for Fas-mediated apoptosis induction by exposure to chemotherapeutics.[10-12] Both the (extrinsic) Fas-mediated apoptosis pathway and the (intrinsic) mitochondrial apoptosis pathway can be targets for modulation by cytotoxic drugs. Combining chemotherapy and Fas-targeted agents may therefore be an interesting method to enhance therapeutic efficacy.

Aim of this study was to determine the apoptosis-inducing potential of APO010 in a broad panel of human solid cancer cell lines, and to assess its ability to circumvent resistance to chemotherapy or to the death ligand TRAIL. In addition, we assessed the potential of combining APO010 with platinum-based chemotherapy.

METHODS

CELL LINES

APO010 sensitivity was determined in three different resistance models (**Table 1**). The human cancer cell lines used for the cisplatin resistance model included the small cell lung cancer (SCLC) cell line GLC_4 and the cisplatin resistant subline (GLC_4-CDDP), the testicular germ cell tumor (TGCT) cell line NTera2/D1 (Tera) and the cisplatin resistant subline (Tera-CP), and the ovarian cancer cell line A2780 and the cisplatin resistant subline (A2780-CP70).[12-15] Two other GLC_4 sublines (GLC_4-Adr and GLC_4-Pgp), both overexpressing ATP-binding cassette (ABC) transporters, comprised the drug-efflux pump mediated resistance model. GLC_4-Adr is an endogenous MRP1 overexpressing cell line, while GLC_4-Pgp has been stably transfected with the *MDR1* gene resulting in overexpression of P-glycoprotein (Pgp).[16,17] The TRAIL resistance model consisted of a recombinant human TRAIL (rhTRAIL) resistant human colorectal cancer (CRC) cell line (SW948-TR) and its parental cell line (SW948).[18]

The SCLC and TGCT cell lines were cultured in RPMI 1640 supplemented with 10% fetal calf serum (FCS), to which 2 mM L-glutamine was added to culture the ovarian cancer cell lines. Both CRC cell lines were cultured in myeloma and Leibovitz medium (1:1), supplemented with 10% FCS.

CHEMICALS, MEDIA, ANTIBODIES AND REAGENTS

Trizol Reagent, M-MLV reverse transcriptase and minimal essential medium (MEM) supplemented with Earle's salts and L-glutamine, were purchased from Invitrogen (Belgium). Propidium iodide, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) and Tween-20 were obtained from Sigma-Aldrich (the Netherlands). DNase-I, POD and Lumi-Light^{plus} were obtained from Roche Diagnostics (Germany),

Table 1. Cell line panel.

	Cell line	Origin	Resistance	(RF)[a]
Cisplatin resistance model	GLC_4	SCLC		
	GLC_4-CDDP	SCLC	cisplatin	(16x)
	Tera	TGCT		
	Tera-CP	TGCT	cisplatin	(2.7x)
	A2780	EOC		
	A2780-CP70	EOC	cisplatin	(5x)
Drug-efflux pump mediated resistance model	GLC_4	SCLC		
	GLC_4-Adr	SCLC	doxorubicin	(330x)
	GLC_4-Pgp	SCLC	paclitaxel, vincristin, doxorubicin	(10x)
TRAIL resistance model	SW948	CRC		
	SW948-TR	CRC	rhTRAIL	(not achieved)[b]

Abbreviations: SCLC, small cell lung cancer; EOC, epithelial ovarian cancer; TGCT, testicular germ cell tumor; CRC, colorectal cancer; RF, resistance factor; (rh)TRAIL, (recombinant human) TNF-related apoptosis inducing ligand.

[a] Except for the intrinsically resistant cell line GLC_4-Pgp, cell lines have been rendered resistant to the indicated drug. The resistance factor (RF) is based on IC50 values.

[b] The IC50 for the rhTRAIL resistant SW948-TR cell line could not be achieved.

doxorubicin-HCl from Pharmachemie (the Netherlands), Ac-DEVD-AFC from Tebu-Bio (the Netherlands), PE-labeled anti-human Fas DX2 monoclonal antibody from BD Biosciences (Belgium), SYBR Green Supermix from BioRad (the Netherlands), mammalian-protein extraction reagent (M-PER) from Pierce (Ireland), protease and phosphatase inhibitors from Thermo Scientific (the Netherlands) and skim milk powder was purchased from Fluka (Switzerland). APO010 was provided by Apoxis (Switzerland). Murine anti-human FADD monoclonal antibody was purchased from BD Biosciences, murine anti-human c-FLIP monoclonal antibody from Alexis Biochemicals (Omnilabo International, the Netherlands), murine anti-human caspase-8 monoclonal antibody from Cell Signaling Technology (Bioké, the Netherlands) and murine anti-human β-actin monoclonal antibody was obtained from ICN Biomedicals (the Netherlands). Horseradish peroxidase (HRP)-conjugated secondary antibodies were obtained from Dako (Denmark).

Survival assay

The cytotoxic effect of APO010 was assessed using MTT assays as described previously.[19] Cells were incubated in a humidified environment for 4 days at 37 °C and 5% CO_2 with APO010 concentrations ranging from 0.1 to 200 ng/ml. MTT (5 mg/ml) was added after the 4-day culture period and formazan crystal production was measured. IC50 values were determined from the survival curves.

APOPTOSIS ASSAY

Cells were cultured in 96-wells plates and exposed to APO010 concentrations up to 50 ng/ml for 24 h. Acridine orange was added subsequently. Apoptosis was defined as the appearance of apoptotic bodies and/or chromatin condensation, using a fluorescence microscope. Results were expressed as the percentage of apoptotic cells in a culture by counting at least 200 cells per well.

To determine if platinum-based chemotherapy sensitizes human cancer cells to Fas-mediated apoptosis induction, apoptosis assays were also performed using a combination of APO010 and cisplatin. These experiments were performed on cell lines comprising the cisplatin resistance model. Cells were pre-exposed to cisplatin (0-20 µM) for 24 h and subsequently received an additional 4 h treatment with cisplatin or cisplatin combined with APO010 (100 ng/ml).

ACTIVATED CASPASE-3 ASSAY

Activated caspase-3 assays were carried out in 96-wells plates as previously described.[20] Cells were exposed to 100 ng/ml APO010 for 4 h and subsequently lysed. Lysate samples were incubated for 1 h at 37 °C with the fluorescence peptide substrate Ac-DEVD-AFC and fluorescence from free 7-amino-4-trifluoromethyl coumarin (AFC) was subsequently measured in a FL600 Fluorometer BioTek plate reader (Beun de Ronde, the Netherlands), using 380 nm excitation and 508 nm emission wavelengths. Caspase-3 activity was determined according to the manufacturer's instructions. Relative caspase-3 activity was calculated by dividing the fluorescence of a sample of treated cells by a sample of untreated cells.

FLOW CYTOMETRY

To determine Fas membrane expression, cells were harvested from the culture medium by centrifugation at 110 x g for 5 min and washed twice with cold phosphate buffered saline (PBS: 6.4 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.14 mM NaCl, 2.7 mM KCl, pH = 7.2) supplemented with 2% FCS and 0.1% sodium azide. Cells were then incubated on ice for 1 h in the dark with the PE-labeled anti-human Fas DX2 monoclonal antibody, which was diluted 10-fold in cold PBS supplemented with 2% FCS and 0.1% sodium azide. Following incubation, cells were washed twice with cold PBS. Analysis was performed on a Coulter Elite Flow cytometer (Becton Dickinson, USA) with Winlist and Winlist 32 software (Verity Software House Inc., USA). Fas membrane levels, calculated

as mean fluorescence intensity (MFI), were determined by comparison of the fluorescence intensity with the fluorescence intensity of isotype controls.

Table 2. RT-PCR primers.

Gene	Primer	5'-sequence-3'	Primer length (BP)	Product length (BP)	Optimal T_{ann} (°C)
Fas	sense	CATGGCTTAGAAGTGGAAAT	20	338	58
	antisense	ATTTATTGCCACTGTTTCAGG	21		
FADD	sense	AGCTCAAAGTCTCAGCACACC	21	250	58
	antisense	TCTGAGTTCCATGACATCGG	20		
Caspase-8	sense	CTGCTTCATCTCTGTATCC	19	355	58
	antisense	GCAAAGTGACTGGATGTACC	20		
c-$FLIP_S$	sense	GAACATCCACAGAATAGACC	20	172	58
	antisense	GTATCTCTCTTCAGGTATGC	20		
c-$FLIP_L$	sense	GAACATCCACAGAATAGACC	20	262	58
	antisense	TTTCAGATCAGGACAATGGG	20		
GAPDH	sense	CACCACCATGGAGAAGGCTGG	21	200	50-65
	antisense	CCAAAGTTGTCATGGATGACC	21		

Abbreviations: BP, base pairs; T_{ann}, annealing temperature; FADD, Fas-associated death domain; c-FLIP, cellular Flice-inhibitory protein; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

REAL-TIME REVERSE TRANSCRIPTION PCR (RT-PCR)

After harvesting exponentially grown cells, pellets were resuspended in 5 x 10^6 cells/ml of Trizol Reagent and stored until use at -80 °C. RNA isolation and subsequent synthesis of complementary DNA (cDNA) was performed as described previously.[19] Total RNA was purified using the RNeasy mini Kit (Qiagen, the Netherlands) according to the manufacturer's instructions, including the optional on-column DNase I digestion to remove trace amounts of DNA contamination. Yield and quality of the total purified RNA was assessed using spectrophotometer absorbance at 230, 260 and 280 nm (with 1 OD unit considered equal to 40 µg/ml) and visualization of 18S and 28S rRNA bands with RNA gel electrophoresis.

Table 2 shows the sequences of the primers used in the quantitative RT-PCR assay. For c-FLIP both isoforms (c-$FLIP_{L(ong)}$ and c-$FLIP_{S(hort)}$) were assessed separately. A gradient RT-PCR was performed to assess primer specificity and to optimize the annealing temperature (T_{ann}) for each set of gene specific primers. Quantitative RT-PCR was performed in 96-wells optical plates (BIOplastics, the Netherlands) using the iCycler iQ™ real-time PCR detection system (BioRad), as previously described.[19] To correct for differences in the amount of starting cDNA between samples, the *GAPDH gene* served as a housekeeping reference gene.

WESTERN BLOTTING

Protein for Western blot analysis was extracted from cell lysates using M-PER according to the manufacturer's instructions. Protease and phosphatase inhibitors were added to the reagent mixture (1:100). Protein yield was determined according to the Bradford assay.[21] Samples were diluted 1:1 with sodium dodecyl sulphate (SDS) sample buffer, containing 0.125 M Tris-HCl, 2% SDS, 10% glycerol, 0.001% bromophenol blue and 10% β-mercaptoethanol, boiled for 5 min and subsequently stored at -20 °C. Proteins (30 µg) were separated on SDS-polyacrylamide gels and transferred onto polyvinylidene difluoride (PVDF) membranes by wet blotting as previously described.[19] Blots were incubated with monoclonal primary antibodies recognizing epitopes on human caspase-8 and human FADD for 1 h at room temperature, or overnight at 4 °C with anti-human c-FLIP antibody. Anti-β-actin antibody served to assure equal protein loading. Membranes were incubated with HRP-labeled secondary antibodies for 1 h at room temperature. Protein bands were visualized with chemiluminescence using POD or Lumi-Light^{plus}.

STATISTICAL ANALYSIS

Unpaired, double-sided Student's *t*-tests were used to determine statistically significant differences in cell survival, apoptosis induction and gene expression between cell lines. *P*-values < 0.05 were considered statistically significant.

RESULTS

APO010 REDUCES CELL SURVIVAL BY INDUCING APOPTOSIS IN VARIOUS SOLID CANCER CELL LINES

The cisplatin sensitive A2780 and resistant A2780-CP70 ovarian cancer cell lines showed decreased cell survival after APO010 exposure, with the parental cell line being more sensitive to APO010 than its resistant counterpart ($IC50_{A2780}$ = 1.4 ng/ml and $IC50_{A2780\text{-}CP70}$ = 4.7 ng/ml) (**Figure 1A**). Both ovarian cancer cell lines showed substantial apoptosis induction after APO010 exposure. No response to APO010 exposure was observed in the other isogenic cisplatin resistance cell line models, neither in the survival assays nor in the apoptosis assays (results not shown).

The drug-efflux pump mediated resistant SCLC cell lines GLC_4-Adr and GLC_4-Pgp both showed decreased survival in response to APO010 exposure compared to the parental GLC_4 cell line (**Figure 1B**). Moderate sensitivity was observed in the GLC_4-Pgp

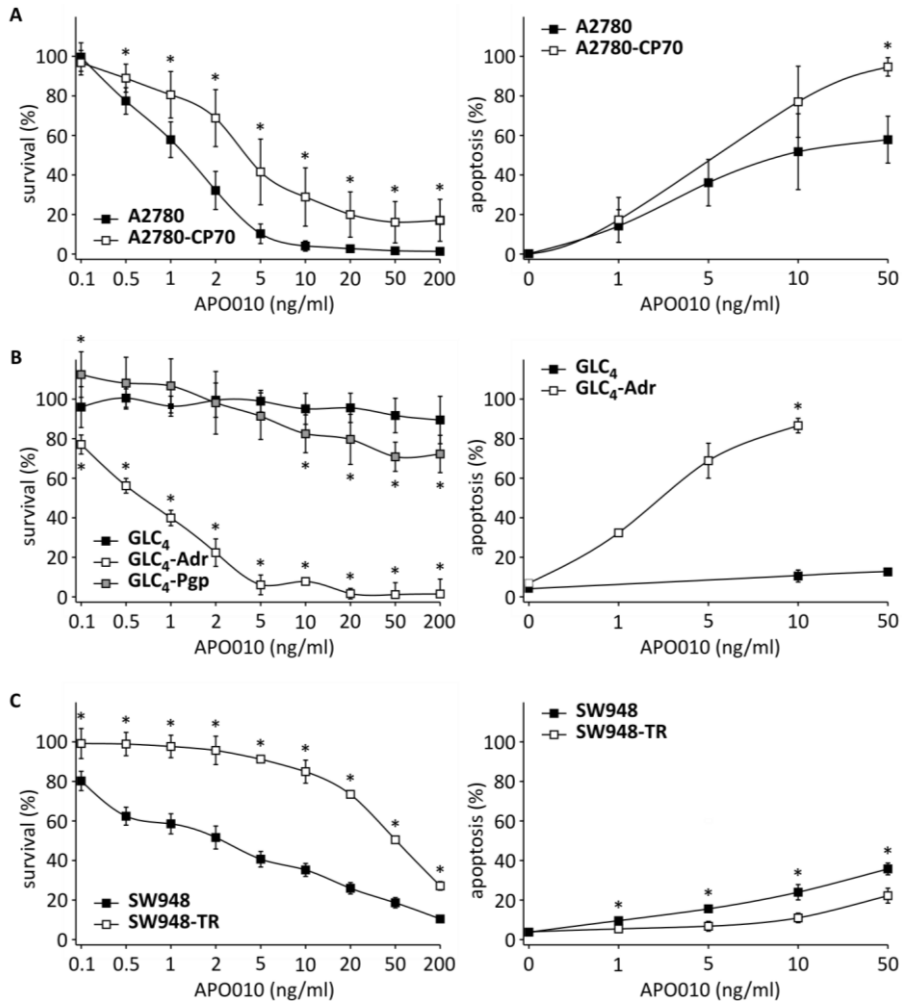

Figure 1. Cytotoxicity was assessed using MTT survival assays, in which cells were incubated for 4 days with APO010 (0.1 to 200 ng/ml). In the apoptosis assays, cells were exposed to up to 50 ng/ml APO010 for 24 h. Data is shown for selected cell lines from the cisplatin resistance model (A), the drug-efflux pump mediated resistance model (B) and the TRAIL resistance model (C), representing the mean ± SD of 3 independent experiments (* indicates $P < 0.05$ compared to the parental cell line).

cell line, whereas the doxorubicin resistant cell line GLC_4-Adr proved extremely sensitive to APO010 ($IC50_{GLC4\text{-}Adr}$ = 0.7 ng/ml). This is supported by the apoptosis assays, showing high levels of apoptosis in the GLC_4-Adr cell line even at low concentrations of APO010.

In the TRAIL resistance model, impairment of cell survival was seen in both the SW948 CRC cell line and its rhTRAIL resistant subline SW948-TR with APO010 (**Figure 1C**). SW948-TR cells, however, were 20-fold less sensitive compared to SW948 cells ($IC50_{SW948}$ = 2.5 ng/ml and $IC50_{SW948\text{-}TR}$ = 53.3 ng/ml). SW948 cells also showed more apoptosis induction upon exposure to APO010 than its resistant counterpart ($P < 0.01$ at all APO010 concentrations used).

EARLY CASPASE-3 ACTIVATION IS SEEN IN APO010 SENSITIVE CELL LINES

Caspase-3 activation was assessed as an early marker of apoptosis after 4 h of APO010 treatment (**Figure 2**). A nearly threefold increase in caspase-3 activation was observed in A2780-CP70 cells after exposure to APO010. A2780, GLC_4-CDDP and GLC_4-Adr cells also displayed enhanced caspase-3 activation, albeit to a lesser extent. No caspase-3 activation was observed in the cell lines that are unresponsive to APO010 (GLC_4, Tera and Tera-CP). In the TRAIL resistance model, an increase in caspase-3 activation was noted in SW948 compared to the resistant subline SW948-TR. As indicated in **Figure 2**, relative caspase-3 activation was seen in those cell lines that were already shown to be sensitive to APO010 exposure in the survival and apoptosis assays, with the exception of SW948-TR.

DETERMINANTS OF APO010 SENSITIVITY

Flow cytometry, RT-PCR analysis and Western blotting were performed to determine expression levels of Fas and components of the DISC (FADD, caspase-8 and c-FLIP), and to relate these parameters to APO010 sensitivity.

Although marked differences were observed in Fas membrane expression between cell lines derived from different tumor types, Fas was detected in all of the cell lines from our panel (**Figure 3A**). Pronounced Fas membrane expression was observed in A2780-CP70, which showed a more than threefold higher expression compared to the already marked expression in A2780 cells. Fas membrane expression was higher in the APO010-sensitive lines GLC_4-CDDP and GLC_4-Adr compared to the parental cell line GLC_4. SW948-TR cells showed lower Fas membrane expression compared to SW948 cells.

The APO010 sensitive GLC_4-Adr cell line exhibits a more than 2-fold higher mRNA expression of Fas compared to the parental APO010 resistant GLC_4 cell line ($P < 0.01$). Compared to the parental cell line, we found lower mRNA expression of Fas in the SW948-TR cell line (0.55 times the expression of SW948 cells, $P < 0.05$).

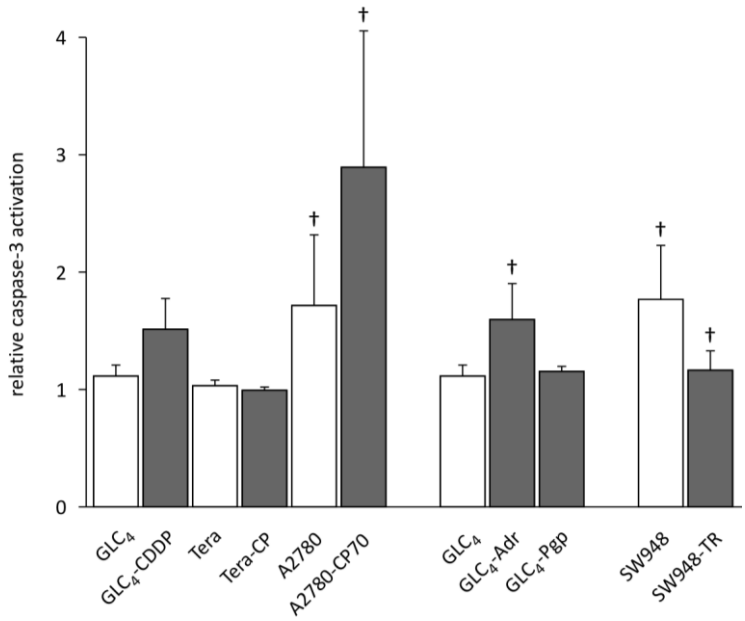

Figure 2. Relative caspase-3 activation after 4 h of exposure to 100 ng/ml APO010. The three groups of bars represent the different cell lines comprising the cisplatin, drug-efflux pump mediated and TRAIL resistance models, respectively. Relative caspase-3 activity was calculated by dividing the fluorescence of a sample of treated cells by a sample of untreated cells. Cell lines that showed sensitivity to APO010 (defined as reaching an IC50 in survival assays plus showing notable apoptosis induction in apoptosis assays) are denoted with †. Data represent the mean ± SD of 3 independent experiments.

Lower caspase-8 protein expression was found in A2780-CP70 cells compared to its parental cell line (**Figure 3B**). Caspase-8 was virtually absent in the APO010 resistant GLC_4-CDDP cells. PCR analyses also demonstrated 5-fold lower caspase-8 mRNA expression in GLC_4-CDDP cells compared to the GLC_4 cell line ($P < 0.01$). The APO010 sensitive cell line SW948 showed higher caspase-8 expression compared to its less sensitive counterpart. c-FLIP protein expression was observed in both ovarian cancer cell lines as well as both CRC cell lines. The other cell lines showed no (all SCLC cell lines) or negligible (the TGCT cell lines) c-FLIP expression.

CISPLATIN UPREGULATES FAS AND SENSITIZES TO APO010

We assessed whether platinum-based chemotherapy could upregulate Fas and thereby sensitize cancer cells to APO010. For this, we used cisplatin sensitive and resistant SCLC (GLC_4 and GLC_4-CDDP) and TGCT (Tera and Tera-CP) cell lines that

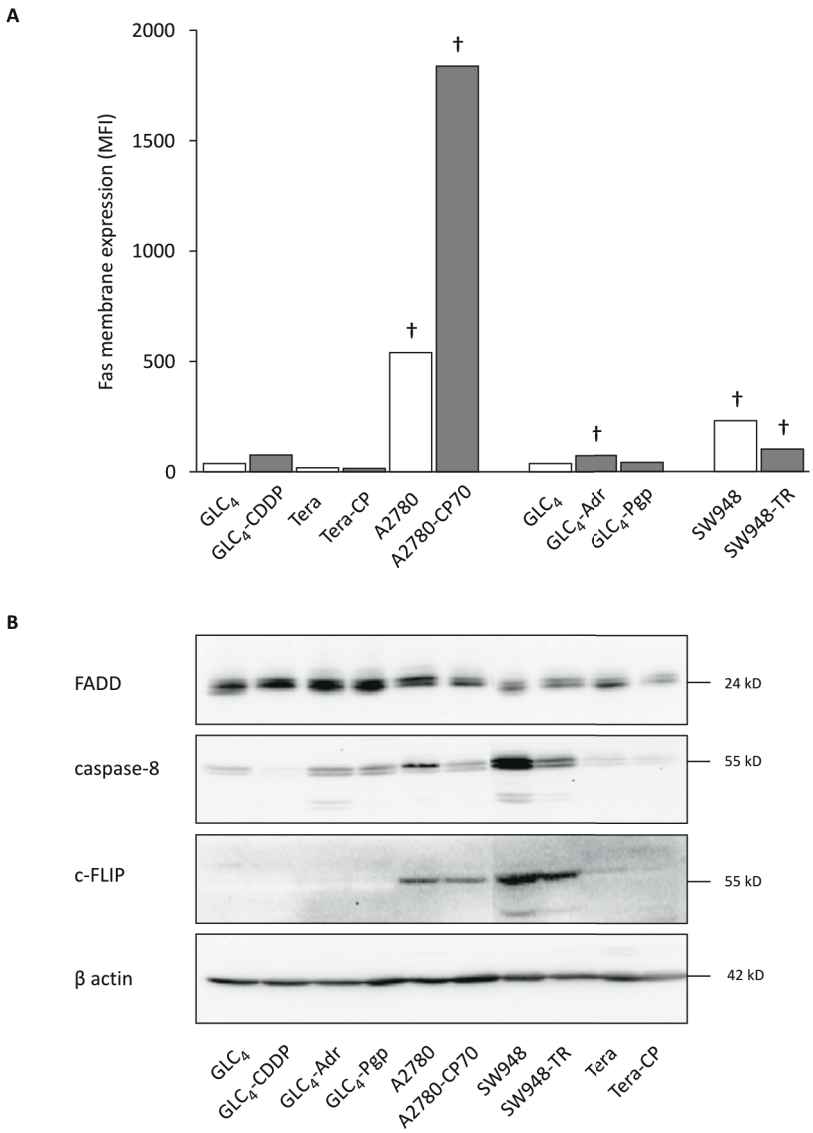

Figure 3. (A) Fas membrane expression of untreated cells expressed as mean fluorescence intensity (MFI), determined by flow cytometry. The three groups of bars represent the different cell lines comprising the cisplatin, drug-efflux pump mediated and TRAIL resistance models, respectively. APO010 sensitive cell lines are denoted with †. Results show a representative experiment. (B) Protein expression of the DISC components FADD, caspase-8 and c-FLIP in untreated cells determined by Western blotting. Actin was used as a loading control.

initially showed no sensitivity to APO010 monotherapy. Cisplatin treatment (10 µM) resulted in a marked increase in membrane-associated Fas in all 4 cell lines (**Figure 4A**). Cisplatin pretreatment sensitized these cell lines to APO010 treatment (**Figure 4B**). Consistently higher rates of apoptosis were seen after treatment with both cisplatin and APO010 compared to single-agent treatment with cisplatin.

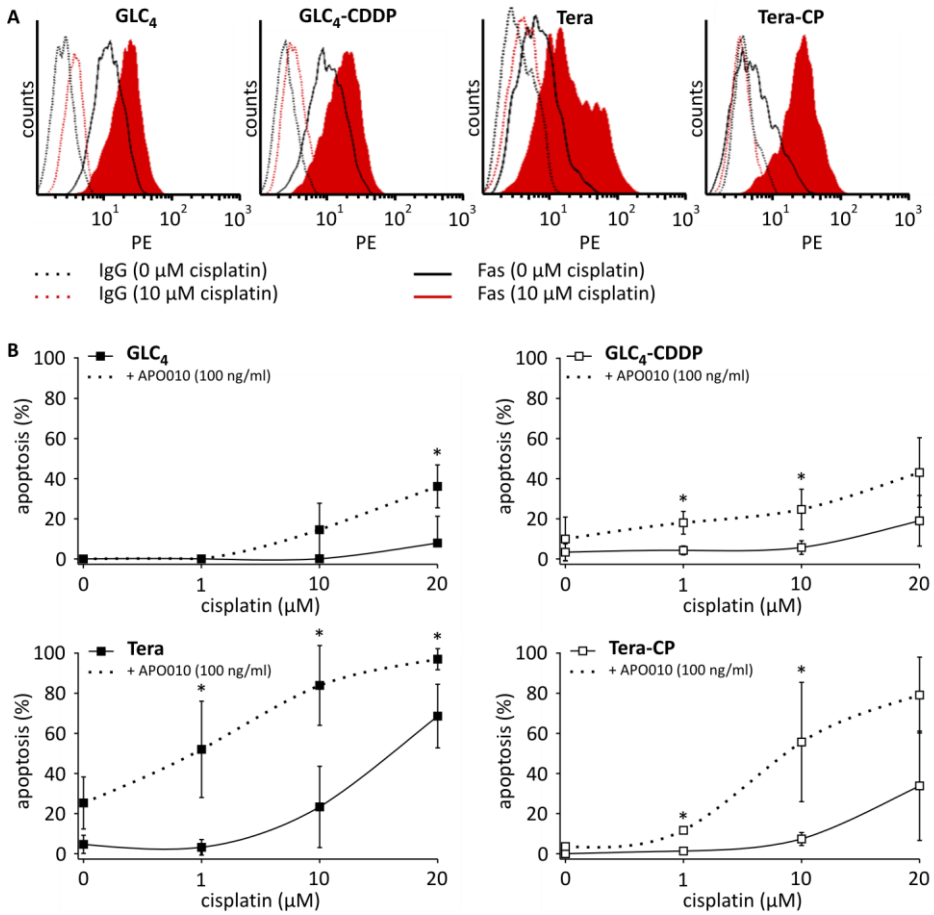

Figure 4. (A) Flow cytometry was performed to assess the potential of cisplatin (10 µM, 24 h incubation) to upregulate Fas membrane expression in cisplatin sensitive and resistant SCLC (GLC_4 and GLC_4-CDDP) and TGCT (Tera and Tera-CP) cell lines, which initially showed no sensitivity to APO010 monotherapy. All cell lines show a clear enhancement of Fas membrane expression. (B) Induction of apoptosis by APO010 after 24 h pretreatment with cisplatin (0-20 µM). Following cisplatin pretreatment, cells were treated for an additional 4 h with cisplatin with or without APO010 (100 ng/ml). Cisplatin sensitizes all cell lines analyzed to APO010-mediated apoptosis induction, independently of platinum resistance. Data represent the mean ± SD of three independent experiments (* indicates $P < 0.05$).

DISCUSSION

This is the first study investigating APO010-mediated apoptosis induction in a large panel of cell lines derived from various solid malignancies. Our study shows substantial differences in APO010 sensitivity, independent of specific inherent chemotherapy or TRAIL resistance. Furthermore, we demonstrate that cisplatin treatment upregulates Fas membrane expression and sensitizes solid cancer cell lines to APO010-induced apoptosis, independent of sensitivity to APO010 monotherapy.

APO010 is a more potent proapoptotic agent than other Fas agonists – *i.e.* agonistic anti-Fas antibodies, sFasL and crosslinked sFasL – in cell lines and primary cells derived from hematological malignancies and human glioma (animal) models.[9,22] APO010 has also been shown to be efficacious in cell lines derived from gastrointestinal stromal tumors (GIST), which generally show abundant Fas expression.[23] In addition, in preclinical ovarian cancer models APO010 was an effective inducer of apoptosis *in vitro* as well as *in vivo*.[24] Our data show sensitivity to APO010 in solid cancer cell lines from various histological backgrounds. Sensitivity to APO010 is independent of cell line specific chemotherapy or rhTRAIL resistance. Previous studies showed that the SCLC cell line GLC_4, both the TGCT cell lines Tera and Tera-CP, and the colon cancer cell line SW948 were resistant to agonistic anti-Fas antibodies.[14,16,25] Similar results were observed with APO010 for GLC_4, Tera and Tera-CP, but not for SW948 cells, suggesting that APO010 can only partially overcome resistance to anti-Fas antibodies.

APO010 exerts its cytotoxic effect by inducing apoptosis via the Fas receptor. Binding of APO010 to membrane-associated Fas induces oligomerization (probably trimerization) of the receptor and subsequent DISC formation. Thus, Fas plays a crucial role in initiating apoptotic signaling and its expression on the cell membrane provides a prerequisite for its activity. Overexpression of Fas in an isogenic glioma model indicated that Fas membrane levels are related to sensitivity to agonistic anti-Fas antibodies.[26] We observed substantial differences in Fas membrane expression between the various solid cancer cell lines comprising our cell line panel. In general, higher Fas membrane expression was observed in APO010 sensitive cell lines compared to their APO010 resistant counterparts. Thus, our results indicate a qualitative relation between the level of receptor expression and APO010 sensitivity. However, we found that Fas membrane expression did not correlate quantitatively with APO010 sensitivity. This is illustrated by the relatively low Fas membrane expression in GLC_4-Adr cells, which showed the highest sensitivity to APO010 amongst

the cell lines tested. Although the importance of Fas membrane expression seems clear, additional downstream signaling components are likely involved in determining (or modulating) APO010 sensitivity.

A threshold mechanism has been established in the Fas-mediated apoptosis pathway in which a certain amount of Fas signaling is required to induce apoptosis.[27] This signaling threshold is modulated by DISC formation.[28] Caspase-8 expression has been found to be a key determinant of sensitivity to sFasL and agonistic anti-Fas antibodies.[29-34] Although caspase-8 expression is essential for Fas-mediated apoptosis induction, our results indicate that the absolute amount of caspase-8 expression is not predictive for the sensitivity to APO010 exposure. c-FLIP, which inhibits caspase-8 activation by competitively binding to FADD, was shown to be a key factor in determining the minimal amount of ligand needed to effectively induce apoptosis.[35] It is proposed that at lower concentrations of a Fas-targeting ligand (subthreshold signaling) c-FLIP will associate with the scarcely formed DISCs and inhibit cleavage of procaspase-8 to its active isoform. Since c-FLIP has a higher DISC binding-affinity, it is able to block Fas-mediated apoptosis signaling until the threshold ligand concentration is achieved.[35,36] In several studies the importance of c-FLIP in Fas-mediated apoptosis has been demonstrated by either overexpression or downregulation of c-FLIP.[37-40] Also, sensitization to APO010 was demonstrated in Bcl-2 overexpressing Jurkat cells in response to diverse anticancer regimens (including ionizing radiation and chemotherapeutic drugs) which commonly downregulated c-$FLIP_{S/L}$ protein levels.[41] c-FLIP RNA interference showed similar results, demonstrating the mechanistic relevance of c-FLIP as a determinant of APO010 sensitivity. Our study indicated that c-FLIP expression does not predict which cell line will show sensitivity to APO010-induced apoptosis, since cell lines that show little c-FLIP expression (*e.g.* GLC_4 and sublines) do not invariably show sensitivity to Fas-mediated APO010 apoptosis signaling. In addition, the APO010 sensitive ovarian and CRC cell lines expressed the highest levels of c-FLIP.

The ratio of caspase-8 and c-FLIP might be important as well. In two recent studies we have investigated the importance of the ratio between caspase-8 and c-FLIP with regard to TRAIL sensitivity. In ovarian cancer cells, caspase-8 overexpression (but not c-FLIP downregulation) resulted in enhanced TRAIL sensitivity, while caspase-8 downregulation diminished TRAIL sensitivity. This indicated that the caspase-8 level was an important determinant.[19] In the CRC cells, caspase-8 downregulation reduced sensitivity while c-FLIP downregulation enhanced TRAIL sensitivity suggesting that the

caspase-8/c-FLIP ratio was important in this model.[18] Since we have not quantitatively determined signaling threshold concentrations for APO010-induced apoptosis, answers to these questions should be provided by future research. Furthermore, the possible influence of downstream factors (such as XIAP) on Fas-mediated apoptosis signaling should be taken into account.[3,42]

Our results show sensitization to APO010-induced apoptosis after pretreatment with platinum-based chemotherapy. Sensitization to Fas-mediated apoptosis can be induced by upregulation of Fas after treatment with various chemotherapeutic drugs, which has been observed in several cancer cell lines including the TGCT cell lines Tera and Tera-CP.[14,28,43,44] Upregulation of Fas has also been described in (senescent) MCF7 breast and A549 lung cancer cells as a consequence of NF-κB-dependent secretion of TNF-α and interferon-γ (IFN-γ).[45] Chemotherapeutic drugs like cisplatin can also lead to activation of NF-κB, which might explain sensitization to APO010 via Fas upregulation. We demonstrated a substantial induction of membrane-associated Fas after cisplatin treatment in the TGCT cell lines as well as GLC_4 and GLC_4-CDDP. Chemotherapy-induced redistribution of Fas into membrane lipid rafts has also been described, promoting receptor clustering and subsequent activation in addition to altering its expression patterns.[28,46] Our results imply Fas upregulation upon platinum-based chemotherapy as an important mechanism of sensitization.

The therapeutic window for APO010, in particular with regard to its toxicity on normal tissues, will eventually determine the applicability of this drug in the clinic.[47] Although anti-Fas antibodies were shown to induce severe hepatotoxicity in mice[4], no severe or irreversible toxicity was observed after cisplatin and APO010 combination treatment in an ovarian cancer xenograft model.[24] It is postulated that the relatively short half-life of APO010 causes less toxicity compared to anti-Fas antibody treatment. However, the toxicity and antitumor efficacy of APO010 need to be assessed more extensively in animal models, comparing both systemic and local APO010 administration. The potential of chemotherapeutic drugs like cisplatin to sensitize cancer cells to APO010 can aid in lowering the amount of APO010 needed for effective apoptosis induction, thereby also limiting its potential cytotoxic effect on normal tissues. The maximum tolerated dose (MTD) for future clinical trials is currently being established in patients with advanced refractory solid cancers.

Our results suggest potential efficacy of APO010 in a broad spectrum of solid malignancies. Due to the lack of cross-resistance between APO010 and conventional

chemotherapeutic drugs, APO010 could be used to circumvent chemoresistance. Upregulation of Fas upon platinum-based chemotherapy provides a rationale for combining these therapies to enhance the antitumor efficacy of APO010.

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CHAPTER 7

SUMMARY, DISCUSSION AND FUTURE PERSPECTIVES

SUMMARY

Ovarian cancer has the highest mortality rate amongst the gynecological malignancies.[1] Patients usually present with advanced stage disease, in which the cancer has already spread beyond the ovaries.[2] This late presentation is largely due to the aspecific nature and late onset of the symptoms accompanying ovarian cancer, like abdominal bloating or pain and common digestive tract complaints like constipation. Attempts to develop early detection strategies have been unsuccessful to date. The prognosis faced by ovarian cancer patients is an inevitable consequence of having advanced stage disease at the time of diagnosis, but the widespread occurrence of chemoresistance is also a major contributor to poor patient outcome.[2] Initial response rates to standard carboplatin *plus* paclitaxel following surgical debulking can be as high as 80%, with 40-60% designated as complete responses, but platinum resistant recurrences often develop.[3] Altogether, this results in a disappointing 25-30% 5-year survival for patients with advanced ovarian cancer.

Finding ways of improving therapeutic efficacy in ovarian cancer has been avidly pursued. Numerous chemotherapeutic regimens have been explored, as well as intraperitoneal administration of chemotherapy, but impact on patient survival has been limited.[4] Consequently, these efforts have not brought much change to the treatment algorithm since the introduction of the current frontline regimen over a decade ago. Since chemotherapy efficacy seems to have reached its ceiling in ovarian cancer, interest has shifted towards the application of molecular targeted drugs to interfere with hallmark biological phenomena crucial to ovarian cancer development and progression. Amongst these, the capacity of ovarian cancer cells to form a supportive vascular system (angiogenesis) and to evade programmed cell death (apoptosis) provide important pharmacological targets for therapy.[5]

An important hurdle for the implementation of molecular targeted drugs in ovarian cancer treatment is posed by its heterogeneity. There are no generic driver mutations present in ovarian cancer which can be universally targeted, and the genetic and molecular profiles differ markedly amongst ovarian cancers.[6] As a consequence, only a selected subgroup of ovarian cancer patients may benefit from using a specific targeted agent. This notion is backed up by ample evidence from clinical trials which explored the value of different targeted agents in ovarian cancer, demonstrating modest response rates and limited survival benefits. It is therefore likely that the implementation of targeted agents in ovarian cancer could benefit from the discovery of patient selection strategies. This could be facilitated by finding upfront predictive

biomarkers or early predictive biomarkers to predict drug efficacy during treatment. Understanding the mechanisms that determine sensitivity or resistance to these agents is therefore of importance.

This thesis describes studies with antiangiogenic and proapoptotic targeted agents in preclinical ovarian cancer models and analysis of potential predictive biomarkers in human ovarian cancers.

Following a short introduction and outline of this thesis in **Chapter 1**, the first chapters are devoted to angiogenesis regulation and inhibition in ovarian cancer. Ovarian cancers are characterized by extensive vascularization and are considered highly angiogenic. Angiogenesis is required for locoregional tumor growth, but also facilitates metastasis.[7] Since angiogenesis is a crucial contributor to tumor development and progression, interest has turned to the development of drugs that inhibit this process.

In **Chapter 2**, an overview is presented on the currently available clinical experience with antiangiogenic therapies in ovarian cancer. The majority of the antiangiogenic agents that have been evaluated in clinical trials target members of the vascular endothelial growth factor (VEGF) family of proangiogenic cytokines or their receptors (VEGFRs), which are regulators of angiogenesis.[8] Ovarian cancer cells produce VEGF ligands (VEGF-A, VEGF-B, VEGF-C, VEGF-D and placental growth factor). These can distinctly activate three receptor tyrosine kinases (VEGFR1, VEGFR2, VEGFR3) present on the cell membrane of vascular endothelial cells, promoting their proliferation, migration and survival. VEGF(R)-targeted agents that have been evaluated thus far in ovarian cancer include the VEGF-A-neutralizing antibody bevacizumab, the VEGFR1/2 fusion protein VEGF-Trap and several small-molecule tyrosine kinase inhibitors (TKIs) capable of inhibiting VEGFR kinase activity. Available experience from phase II and III clinical trials is summarized in this chapter. Although these agents show some activity when incorporated into frontline therapy and when given as monotherapy for recurrent disease, response rates and survival gains are too modest to justify their use in general clinical practice.[9] This underlines the need for patient selection strategies and has fuelled a search for alternative antiangiogenic therapies. Biomarker data as well as preclinical data obtained with alternative angiogenesis inhibitors are also described in **Chapter 2**.

Some of the clinical trials published to date have included exploratory biomarker analyses. However, the number of patients included in these analyses has been small and circulating biomarkers have been the focus of attention.[10,11] Currently, no markers have been discovered to aid the selection of patients which are likely to respond to VEGF(R)-targeted drugs. Insight into the expression of multiple vascular endothelial growth factor (VEGF) family members can support the implementation of antiangiogenic therapy, considering that these cytokines are the main therapeutic targets. In **Chapter 3**, VEGF family member expression was studied in ovarian cancers and their related omental metastases. Tissue microarrays (TMAs) encompassing 270 primary cancers and 112 paired metastases were immunostained for VEGF-A, VEGF-B, VEGF-C and VEGF-D. Protein expression profiles were related to clinicopathological characteristics and survival. Immunohistochemical positivity was observed for VEGF-A in 90%, VEGF-B in 4%, VEGF-C in 41% and VEGF-D in 55% of the primary ovarian cancers. VEGF-A expression correlated with VEGF-C and VEGF-D expression. Simultaneous positivity for VEGF-A and VEGF-C was observed in 38% of the cancers, and for VEGF-A and VEGF-D in 54%. Metastases showed positivity for VEGF-A in 78%, VEGF-B in 5%, VEGF-C in 26% and VEGF-D in 45% of cases. VEGF family member expression was independent of common clinicopathological parameters and showed no independent prognostic significance in multivariate analysis. The frequent simultaneous expression of VEGF-A, VEGF-C and VEGF-D in ovarian cancers observed may contribute to bevacizumab resistance. It has been previously demonstrated that prolonged exposure of colorectal cancer and glioma cells to bevacizumab results in compensatory upregulation of VEGF-C and VEGF-D which stimulate the proliferation of endothelial cells despite effective VEGF-A blockade, highlighting their potential role in bevacizumab resistance.[12,13] Combinatorial analysis of VEGF family member expression could support a rational, individualized choice of antiangiogenic therapy and might be used to predict antiangiogenic drug efficacy.

Several molecular targeted agents are being evaluated for their antiangiogenic potential. In ovarian cancer, inhibitors of the mammalian target of rapamycin (mTOR) provide an interesting alternative to the aforementioned VEGF(R)-targeted agents.[14] The serine/threonine kinase mTOR controls the translation of several proteins which contain specific regulatory sequences in their messenger RNA (mRNA), including VEGF-A and its transcriptional regulator hypoxia inducible factor-1α (HIF-1α).[15] Inhibitors of mTOR, rapamycin (sirolimus) and its analogs (rapalogs, like everolimus) can thus

potentially reduce VEGF-A production by ovarian cancer cells. Changes in tumor VEGF-A levels might be used as an early read-out for response to these agents. If so, longitudinal monitoring of tumor VEGF-A levels would greatly facilitate response assessment early on after starting treatment with a mTOR inhibitor. Positron emission tomography (PET) using radiolabeled bevacizumab provides a tool for non-invasive monitoring of tumor VEGF-A levels in response to treatment.[16] In **Chapter 4**, we investigated whether early changes in tumor VEGF-A levels after everolimus treatment could be monitored with ^{89}Zr-bevacizumab PET in mice. First, we showed a decrease in VEGF-A secretion by everolimus in a panel of ovarian cancer cell lines. BALB/c mice were subsequently xenografted subcutaneously with A2780^{luc+} ovarian cancer cells and were given daily everolimus (10 mg/kg intraperitoneally) for 14 days. ^{89}Zr-bevacizumab PET scans were performed before (baseline) and after treatment, from which mean standardized uptake values (SUV_{mean}) were calculated. For *ex vivo* analyses, control animals were sacrificed after the baseline scans. Everolimus treatment decreased ^{89}Zr-bevacizumab uptake by 21.7 ± 4.0% (SUV_{mean} 2.26 ± 0.18 *versus* 2.89 ± 0.20, $P < 0.01$). *Ex vivo* biodistribution confirmed decreased tumor uptake of the tracer in treated animals compared to control animals (7.78 ± 0.84 %ID/g *versus* 14.02 ± 1.68 %ID/g, $P < 0.01$), while no differences were observed in other tissues. VEGF-A protein expression was indeed lowered in treated versus untreated tumors ($P < 0.05$), which resulted in a diminished vascular density. Our data show that ^{89}Zr-bevacizumab PET can visualize and quantify reduced tumor VEGF-A levels in response to everolimus therapy. We therefore propose clinical testing of ^{89}Zr-bevacizumab PET as an early predictor of antiangiogenic efficacy in response to mTOR inhibitor therapy.

Chapter 5 and **Chapter 6** are devoted to *in vitro* studies assessing the potential of death ligand-based therapies targeting the extrinsic apoptosis pathway. We studied the apoptosis inducing potential of two drugs related to members of the tumor necrosis factor (TNF) superfamily of death ligands: trimeric recombinant human TNF-related apoptosis-inducing ligand (rhTRAIL) and a hexameric form of Fas ligand (MegaFasL, or APO010).

Like TNF, rhTRAIL and APO010 are capable of binding to death receptors (DRs) present on the outer membrane of ovarian cancer cells. Upon binding of TRAIL to either TRAIL receptor 1 (DR4) or 2 (DR5), or binding of APO010 to Fas, the extrinsic apoptosis pathway is activated via assembly of a membrane-associated death inducing signaling complex (DISC).[17] The DISC is a multiprotein complex, encompassing the

death receptor, the adaptor molecule Fas-associated death domain (FADD), the proapoptotic signal transduction protein (pro)caspase-8 and the inhibitory protein cellular Flice-like inhibitory protein (c-FLIP). Cleavage of procaspase-8 to its active form initiates the caspase cascade making up the extrinsic apoptosis pathway. Whether or not apoptosis is induced depends on the balance between pro- and antiapoptotic signals.

Triggering apoptosis using death ligands like rhTRAIL might overcome the problem of chemoresistance in ovarian cancer. *In vitro*, conventional chemotherapeutic drugs can act synergistically with death receptor-targeted agents, enhancing cell death in both chemosensitive and -resistant ovarian cancer cell lines.[18,19] The mechanisms underlying the sensitization to rhTRAIL by conventional chemotherapy can be best studied in isogenic cell line models with differential chemosensitivity. In **Chapter 5** we aimed to find molecular determinants of rhTRAIL sensitivity in an isogenic ovarian cancer cell line model of platinum resistance as well as the mechanism of synergy between rhTRAIL and cisplatin. To this end, we exposed A2780 ovarian cancer cells and their platinum resistant CP70 counterparts to rhTRAIL alone or in combination with cisplatin. A2780 cells showed moderate sensitivity to rhTRAIL-induced apoptosis, while CP70 cells were resistant. Combining rhTRAIL with cisplatin strongly increased levels of apoptosis in both cell lines, which could be explained by elevated caspase-8 protein and mRNA levels upon cisplatin exposure, in particular in CP70 cells. Untreated CP70 cells expressed less caspase-8 protein compared to A2780, while mRNA levels were similar. Caspase-8 mRNA turnover and protein stability did not differ between both cell lines in the presence or absence of cisplatin. By downregulating caspase-8 with small interfering RNA (siRNA) and by using constructs to overexpress caspase-8, we showed that the induction of caspase-8 protein levels by cisplatin is essential for sensitizing ovarian cancer cells to rhTRAIL. Additional c-FLIP and p53 siRNA experiments showed that neither an altered caspase-8/c-FLIP ratio nor a p53-dependent increase in DR5 membrane expression following cisplatin is involved in sensitization to rhTRAIL. We conclude that cisplatin enhances rhTRAIL-induced apoptosis in platinum sensitive and resistant ovarian cancer cells, and that induction of caspase-8 protein expression is the key factor in rhTRAIL sensitization.

In **Chapter 6** we aimed to study the apoptosis-inducing potential of APO010 in 12 human solid tumor cell lines with various resistance profiles, including platinum

sensitive and resistant ovarian cancer cell lines, to assess its ability to circumvent resistance to chemotherapy and death ligands. These cell lines together comprised three isogenic resistance models, in which we determined cell survival, apoptosis induction, caspase-3 activation, Fas membrane expression and the expression of DISC components in a panel of human solid tumor cell lines. Several solid tumor cell lines showed reduced cell survival and apoptosis induction upon APO010 exposure, including A2780 and CP70 ovarian cancer cells. Sensitivity to APO010 was independent of inherent resistance to chemotherapy. Fas membrane expression, determined by flow cytometry, correlated qualitatively with sensitivity to APO010. Fas membrane expression was highest in the ovarian cancer cell lines compared to cell lines derived from other solid cancers. Interestingly, pretreatment with cisplatin resulted in upregulation of Fas membrane expression and sensitization of cancer cells to APO010 independently of inherent cisplatin resistance or initial insensitivity to APO010 monotherapy. These data show that APO010 is a potent inducer of apoptosis in various drug sensitive and resistant solid tumor cell lines, with no consistent cross-resistance observed between APO010 and classical chemotherapeutic agents, indicating APO010 can be used to circumvent chemoresistance. In addition, combining APO010 with platinum-based chemotherapy can enhance its antitumor efficacy.

DISCUSSION AND FUTURE PERSPECTIVES

Because of the marked molecular heterogeneity observed among ovarian cancers, finding tools for patient selection is key to (cost-)effective implementation of molecular targeted agents. Molecular profiling studies have provided evidence that different ovarian cancer subtypes arise from precursor lesions of different histological origin, implying different mechanisms in their carcinogenesis. Differences in (epi)genetic background or (micro)environmental signaling cues may result in activation of specific oncogenic signaling pathways.[20] Targets for therapy are therefore likely subtype-dependent. For example, the presence of a BRCAness profile in serous ovarian cancers renders them more sensitive to PARP inhibitors.[21] Also, inhibitors of mTOR or phosphoinositide-3-kinase (PI3K) seem more potent in clear cell ovarian cancers, which display stronger phosphorylation of Akt.[22] Clear heterogeneity also exists among ovarian cancers with the same histological subtype. This is illustrated by the discovery of three distinct serous ovarian cancer gene expression signatures, characterized mainly by differences in expression of angiogenesis-related genes.[23] High-throughput analyses like deep sequencing, microarrays and proteome/kinome

profiling tools have readily become available and can offer valuable insight into subtype-specific ovarian cancer biology and targeted drug sensitivity. This provides clues as to which molecular targeted therapy certain ovarian cancers might respond best.

Ultimately, however, patient-tailored therapy will rely on the discovery of biomarkers that can be applied at the level of the individual patient. Analyzing upfront target expression in patients receiving molecular targeted agents seems to be a logical first step in the quest for biomarker discovery, and should preferentially be included in every phase II or III clinical trial investigating their efficacy. In this respect, for example, analysis of tumor VEGF-A expression when using antiangiogenic drugs could provide a primary rationale for either giving or withholding therapy. However, as we have demonstrated in **Chapter 5** and **Chapter 6**, numerous other (non-target) factors can be important determinants of sensitivity to targeted drugs. Their relevance in different tumor types may vary, which makes it difficult to pinpoint universal biomarkers that can be used for patient selection. Moreover, adaptive responses in reaction to molecular targeted drugs may influence (initial) sensitivity, as has been demonstrated for bevacizumab which induces compensatory upregulation of VEGF-C and VEGF-D by cancer cells.[12] More preclinical models that reflect the existing biological diversity between and among ovarian cancer histological subtypes are needed to understand tumortype-specific mechanisms responsible for drug sensitivity.

Preclinical studies aimed at unraveling mechanisms of targeted drug resistance might provide insight in potential biomarkers as well as providing a rationale for effective combination therapies. Since the nature of these mechanisms is likely to differ between cytotoxic (*e.g.* proapoptotic) and cytostatic (*e.g.* antiangiogenic) therapies, different experimental approaches and techniques should be adopted in their preclinical investigation.

Exposure to chemotherapeutic or death receptor-targeted drugs will result in the death of ovarian cancer cells sensitive to these treatments, making it likely that clonal selection plays an important role in (acquired) resistance. Genetic or epigenetic screening of cell lines or tissue samples before treatment and after resistance has occurred may therefore be the best initial approach. Data from such studies can be compared to data obtained when analyzing sensitive *versus* primarily insensitive cancers, to assess overlap between intrinsic and acquired resistance.

In the case of other targeted drugs which do not (primarily) induce cell death, like mTOR inhibitors, resistance could occur in the same cells which were initially

sensitive to the study drug. This could be a consequence of rescue or feedback mechanisms activated in response to treatment. Examples of such mechanisms are emerging for many molecular targeted agents. Induction of HIF-1α activity in response to angiogenesis inhibition could paradoxically cause a more aggressive tumor phenotype upon treatment with, for example, the VEGFR-targeted TKI sunitinib.[24] Also, for mTOR inhibitors compensatory feedback mechanisms have been described, mainly resulting in Akt activation.[25] In some instances, these feedback mechanisms may induce a state comparable to oncogene addiction, without the need for activating mutations or other underlying (epi)genetic aberrations. Proteomic or kinomic analyses might serve to understand (acquired) resistance to cytostatic molecular targeted therapy. Creation of isogenic models for resistance to molecular targeted agents, which has also been done in efforts investigating resistance to chemotherapy, could be of value. It is of interest to develop inducible knock-out systems for *in vivo* studies to further explore the importance of feedback-mediated drug resistance, for example by generating models stably transfected with tetracycline-inducible insulin receptor substrate-1 (IRS-1) and HIF-1α short hairpin RNA (shRNA) expression vectors.

As upfront biomarkers often fail, it is of interest to see whether the early effect of the study drug on the tumor can be measured. This can be done with repeat biopsies, but this necessitates multiple invasive procedures. An alternative could be to longitudinally collect circulating tumor cells (CTCs), although this does not take into account the influence of interactions between cancer cells and the tumor microenvironment.[26] Molecular imaging has the advantage of providing insight into the actual tumor biology, if the putative biomarker is accessible for tracer binding (*i.e.* membrane- or matrix-associated) or intracellular trapping. ^{89}Zr-bevacizumab is an interesting candidate biomarker, considering that solely blocking VEGF-A with bevacizumab has shown clinical efficacy in ovarian cancer trials (see **Chapter 2**).[27-29] This tracer could be of value as an early predictive biomarker for angiogenesis inhibitors capable of reducing VEGF-A levels, including mTOR inhibitors as demonstrated in **Chapter 4**, or heat shock protein 90 (HSP90) inhibitors. Ongoing clinical studies will provide answers concerning the clinical utility of ^{89}Zr-bevacizumab. Similarly, tracers are being developed to assess DR4/5 expression to select patients for death receptor-targeted therapy.[30]

To conclude, implementation of antiangiogenic and proapoptotic drugs is likely to depend on the discovery of biomarkers for patient selection. Establishing solid biomarkers will rely on understanding ovarian cancer biology and the mechanistic

basis of drug sensitivity and resistance. Therefore, an effort should be made in improving and developing preclinical models that accurately reflect the existing biological diversity between and among ovarian cancer histological subtypes.

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CHAPTER 8

NEDERLANDSE SAMENVATTING

(SUMMARY IN DUTCH)

SAMENVATTING

Van de gynaecologische maligniteiten lijdt eierstokkanker (ovariumkanker) de hoogste mortaliteit.[1] Patiënten met ovariumkanker presenteren zich meestal als de ziekte zich reeds buiten de eierstokken heeft verspreid.[2] Deze late ziektepresentatie komt grotendeels voort uit het laat optreden van (veelal aspecifieke) symptomen, waaronder buikpijn, een opgezet gevoel in de buik en veelvoorkomende buikklachten zoals obstipatie. Tot op heden is het niet gelukt om zinvolle vroegdiagnostiek te ontwikkelen. De slechte prognose van patiënten met ovariumkanker is enerzijds gerelateerd aan de mate van ziekte-uitbreiding ten tijde van de diagnose, anderzijds aan het veelvuldig optreden van chemoresistentie.[2] Hoewel circa 80% van de patiënten respondeert op standaardbehandeling, bestaande uit carboplatine-paclitaxel chemotherapie na chirurgische tumorreductie, treden er vaak recidieven op.[3] Dit resulteert in een teleurstellende 5-jaarsoverleving van 25-30% bij patiënten met een vergevorderd stadium ovariumkanker.

Daarom is er veel aandacht voor het vergroten van de behandeleffectiviteit bij ovariumkanker. Verschillende chemotherapeutische behandelingen zijn onderzocht, inclusief het intraperitoneaal toedienen van chemotherapie, maar de winst in patiëntoverleving is beperkt.[4] Dientengevolge is er weinig veranderd in de behandeling van ovariumkanker sinds de introductie van de huidige standaardbehandeling, inmiddels circa 10 jaar geleden. De effectiviteit van chemotherapie lijkt zijn plafond te hebben bereikt. Daarom is de interesse verlegd naar het gebruik van doelgerichte moleculaire medicijnen ('targeted drugs') waarmee kan worden ingegrepen op biologische sleutelprocessen die cruciaal zijn voor de ontwikkeling en progressie van ovariumkanker. Het vermogen van ovariumkankers om een eigen vaatnetwerk te vormen (angiogenese) en om te ontsnappen aan geprogrammeerde celdood (apoptose) biedt potentieel belangrijke farmacologische aangrijpingspunten voor behandeling.[5]

De biologische heterogeniteit binnen ovariumkankers vormt een belangrijk obstakel bij de implementatie van doelgerichte moleculaire medicijnen. Er zijn geen alomtegenwoordige DNA mutaties bekend die een aanknopingspunt vormen voor behandeling, en de genetische en moleculaire profielen verschillen aanzienlijk tussen verschillende ovariumkankers.[6] Zodoende is het aannemelijk dat slechts een selecte subgroep patiënten mogelijk baat heeft bij een bepaalde doelgerichte therapie. Dit wordt ondersteund door de resultaten van vele klinische studies waarin de waarde van uiteenlopende doelgerichte medicijnen is onderzocht in ongeselecteerde groepen

patiënten met ovariumkanker, met veelal geringe effectiviteit en minimale overlevingswinst als uitkomst. Om de waarde van doelgerichte moleculaire behandelingen vast te kunnen stellen, is het daarom van belang enkel die patiënten te selecteren die (waarschijnlijk) baat hebben bij een specifieke doelgerichte therapie. Dit kan met predictieve biomarkers die selectie van patiënten voorafgaand aan een behandeling mogelijk maken, of met vroegpredictieve biomarkers die de uiteindelijke effectiviteit al kort na het starten van een behandeling voorspellen. Voor het identificeren van dergelijke biomarkers is een gedegen begrip van de mechanismen die gevoeligheid of resistentie voor de verscheidene doelgerichte moleculaire medicijnen bepalen onontbeerlijk.

Dit proefschrift beschrijft onderzoek verricht met vaatgroeiremmende (antiangiogene) en geprogrammeerde celdood-inducerende (proapoptotische) medicijnen in preklinische ovariumkankermodellen, en evaluatie van potentiële predictieve biomarkers in ovariumkanker weefsels.

Na een korte introductie en beschrijving van de inhoud van het proefschrift in **Hoofdstuk 1**, zijn de **Hoofdstukken 2-4** gewijd aan de regulatie en remming van angiogenese in ovariumkanker. Ovariumkankers worden gekarakteriseerd door uitgebreide vaatvorming. Angiogenese is noodzakelijk voor locoregionale tumorgroei, maar speelt tevens een belangrijke rol bij het tot stand komen van uitzaaiingen.[7] Omdat angiogenese een cruciale bijdrage levert aan de ontwikkeling en groei van tumoren, zijn er verscheidene medicijnen ontwikkeld gericht op het remmen van dit proces.

In **Hoofdstuk 2** wordt een literatuuroverzicht gegeven van de beschikbare klinische ervaring met antiangiogene medicijnen in ovariumkanker. De meerderheid van de antiangiogene medicijnen die in klinische studies zijn getest, zijn gericht op het remmen van vasculair endotheliale groeifactoren (VEGFs) of hun receptoren (VEGFRs). Deze spelen een belangrijke rol in de regulatie van angiogenese.[8] Ovariumkankercellen produceren verscheidene VEGFs (VEGF-A, VEGF-B, VEGF-C, VEGF-D en placentaire groeifactor). Deze VEGFs kunnen verschillende membraangebonden receptoren (VEGFR1, VEGFR2, VEGFR3) activeren, die aanwezig zijn op endotheelcellen van zowel bloed- als lymfevaten. Hierdoor worden deze cellen gestimuleerd tot deling, migratie en overleving. De VEGF(R)-gerichte medicijnen tot dusverre onderzocht in ovariumkanker zijn het VEGF-A-neutraliserende antilichaam bevacizumab, het VEGFR1/2 fusie eiwit VEGF-Trap en verscheidene tyrosine kinase remmers (TKIs)

gericht tegen VEGFR kinase activiteit. De beschikbare ervaring uit fase II en III studies met deze medicijnen is samengevat in **Hoofdstuk 2**. De VEGF(R)-gerichte medicijnen verlengen de ziektevrije overleving met enige maanden wanneer ze worden geïncorporeerd in de huidige standaardbehandeling of wanneer ze worden gegeven als monotherapie bij recidief ziekte. De overlevingswinst is over het algemeen te gering om de toepassing van deze medicijnen in de klinische praktijk te rechtvaardigen.[9] Dit onderstreept de noodzaak voor patiëntselectie en stimuleert de zoektocht naar alternatieve antiangiogene behandelingen. Tevens worden in **Hoofdstuk 2** de beschikbare data omtrent potentiële biomarkers en data uit preklinische studies met alternatieve angiogeneseremmers samengevat.

In een deel van de tot op heden gepubliceerde klinische studies met angiogeneseremmers zijn biomarkeranalyses opgenomen. Echter, deze studies zijn van beperkte omvang en er is met name gekeken naar circulerende biomarkers.[10,11] Op dit moment zijn er geen markers ontdekt die helpen bij de selectie van patiënten die mogelijk baat kunnen hebben bij VEGF(R)-gerichte medicijnen. Inzicht in de expressie van de verschillende VEGFs kan mogelijk de implementatie van antiangiogene therapie bij ovariumkanker bevorderen, aangezien deze eiwitten – en de receptoren die zij activeren – de aangrijpingspunten vormen voor VEGF(R)-gerichte medicijnen. In **Hoofdstuk 3** is de expressie van eiwitten behorend tot de VEGF familie onderzocht in ovariumkankers en omentummetastasen afkomstig van deze ovariumkankers. Tissue microarrays (TMAs) met daarop weefsel van 270 primaire ovariumkankers en 112 gepaarde metastasen werden hiertoe immunohistochemisch gekleurd op VEGF-A, VEGF-B, VEGF-C en VEGF-D. Eiwitexpressie profielen werden gerelateerd aan klinisch-pathologische karakteristieken en overlevingsgegevens. Expressie werd aangetoond van het VEGF-A eiwit bij 90%, VEGF-B bij 4%, VEGF-C bij 41% en VEGF-D bij 55% van de primaire kankers. VEGF-A expressie correleerde met VEGF-C en VEGF-D expressie. Gelijktijdige positiviteit voor zowel VEGF-A als VEGF-C werd gezien in 38% van de kankers, en voor VEGF-A met VEGF-D in 54%. Metastasen lieten in 78% van de gevallen expressie van VEGF-A zien, in 5% van VEGF-B, in 26% van VEGF-C en in 45% van VEGF-D. De expressie van de verschillende VEGFs bleek onafhankelijk te zijn van gebruikelijke klinisch-pathologische parameters en had geen onafhankelijke prognostische waarde in multivariate analyse. Het frequent gelijktijdig voorkomen van VEGF-A, VEGF-C en VEGF-D in ovariumkankers zou kunnen bijdragen aan resistentie voor bevacizumab. Eerdere studies in darmkanker- en glioomcellen toonden dat langdurige blootstelling aan bevacizumab leidt tot compensatoire opregulatie van VEGF-C en VEGF-D, welke op

hun beurt de deling van endotheelcellen stimuleren ondanks effectieve blokkade van VEGF-A.[12,13] Dit wijst op een potentiële rol van VEGF-C en VEGF-D in bevacizumab resistentie. Gecombineerde analyse van diverse eiwitten uit de VEGF familie kan mogelijk een rationele, geïndividualiseerde keuze van antiangiogene behandeling mogelijk maken, en zou daarmee mogelijk kunnen worden gebruikt om de effectiviteit van antiangiogene behandeling voor de individuele patiënt te voorspellen.

Verschillende doelgerichte medicijnen worden momenteel onderzocht op hun antiangiogene werking. Bij de behandeling van ovariumkanker bieden remmers van het eiwit 'mammalian target of rapamycin' (mTOR) een alternatief voor de in **Hoofdstuk 2** beschreven VEGF(R)-gerichte medicijnen.[14] mTOR is een serine/threonine kinase die de synthese van verscheidene eiwitten met specifieke regulatoire elementen in hun messenger RNA (mRNA) controleert, waaronder VEGF-A en haar transcriptionele regulator hypoxie-geïnduceerde factor-1α (HIF-1α).[15] Farmacologische mTOR remming, met medicijnen als rapamycine (sirolimus) en rapamycine-derivaten (rapalogen, waaronder everolimus), kan daarom in opzet leiden tot verminderde VEGF-A productie door ovariumkankercellen. De verandering in tumor VEGF-A productie, die teweeg wordt gebracht door deze medicijnen, zou daarbij kunnen dienen als een vroege uitlezing voor de respons op deze medicijnen. Longitudinale monitoring van tumor VEGF-A expressie zou daarom een belangrijke bijdrage kunnen leveren aan het beoordelen van de werkzaamheid van mTOR remmers in een vroeg stadium na starten van behandeling. Tumor VEGF-A spiegels kunnen niet-invasief worden afgebeeld en gemeten met positron emissie tomografie (PET), waarbij gebruik wordt gemaakt van bevacizumab waaraan een radio-isotoop is gekoppeld.[16] In **Hoofdstuk 4** hebben we onderzocht of vroege veranderingen in tumor VEGF-A spiegels na behandeling met de mTOR remmer everolimus kunnen worden vervolgd met ^{89}Zr-bevacizumab PET in muizen met een geïmplanteerde tumor. We laten hier ten eerste zien dat everolimus leidt tot een afname van VEGF-A secretie in een panel van ovariumkankercellijnen. Vervolgens werden humane A2780^{luc+} ovariumkankercellen subcutaan geïnjecteerd in BALB/c muizen. Nadat deze cellen waren uitgegroeid tot meetbare tumoren, kregen de muizen dagelijks everolimus (10 mg/kg) intraperitoneaal toegediend gedurende 14 dagen. Voorafgaand aan (baseline) en na behandeling met everolimus werden ^{89}Zr-bevacizumab PET scans gemaakt, waarbij de gemiddelde gestandaardiseerde traceropnamewaarde ($SUV_{gemiddeld}$) werd bepaald. Behandeling met everolimus verminderde de ^{89}Zr-bevacizumab opname met 21.7 ± 4.0% ($SUV_{gemiddeld}$ 2.26 ± 0.18 *versus* 2.89 ± 0.20, $P < 0.01$). *Ex vivo* meting van de

radioactiviteit in tumoren en organen (biodistributie) van behandelde en onbehandelde dieren bevestigde de afname in traceropname (7.78 ± 0.84 %ID/g *versus* 14.02 ± 1.68 %ID/g, $P < 0.01$), terwijl er geen verschillen werden gezien in traceropname door andere weefsels. VEGF-A eiwitexpressie was verlaagd in behandelde *versus* onbehandelde tumoren ($P < 0.05$), wat resulteerde in verminderde vaatdichtheid in behandelde tumoren. Onze data tonen aan dat ^{89}Zr-bevacizumab PET een vermindering in tumor VEGF-A spiegels na behandeling met everolimus kan visualiseren en kwantificeren. Klinische studies zijn noodzakelijk om vast te stellen of ^{89}Zr-bevacizumab PET van waarde is als vroegpredictieve biomarker voor de effectiviteit van mTOR remmers in patiënten met ovariumkanker.

Hoofdstuk 5 en **Hoofdstuk 6** beschrijven *in vitro* onderzoek met 'death ligands' gericht op het activeren van de extrinsieke apoptoseroute en resulterend in geprogrammeerde celdood. Hierbij hebben we gekeken naar de potentie van twee liganden die behoren tot de tumor necrose factor (TNF) superfamilie: trimerisch recombinant humaan TNF-gerelateerde apoptose-inducerende ligand (rhTRAIL) en een hexamerische vorm van Fas ligand (MegaFasL, APO010).

Net als TNF, zijn TRAIL en APO010 in staat 'death' receptoren (DRs) te binden die aanwezig zijn op de celmembraan van ovariumkankercellen. Binding van TRAIL aan TRAIL receptor 1 (DR4) of 2 (DR5), evenals binding van APO010 aan Fas, leidt tot activatie van de extrinsieke apoptoseroute. Deze activatie wordt gemedieerd door het membraan-geassocieerde 'death inducing signaling complex' (DISC).[17] Dit complex bestaat uit meerdere eiwitten, waaronder de DR, het adaptatiemolecuul FADD ('Fas-associated death domain'), het proapoptotische eiwit (pro)caspase-8 en een negatieve regulator, c-FLIP ('cellular FLICE-like inhibitory protein'). Klieving van het procaspase-8 tot actief caspase-8 initieert de caspase cascade, welke deel uitmaakt van de extrinsieke apoptoseroute. Of binding van een TNF-ligand uiteindelijk leidt tot apoptose, is afhankelijk van de balans tussen pro- en antiapoptotische signalen in de cel.

Inductie van apoptose met liganden zoals rhTRAIL kan in potentie het probleem van chemoresistentie bij de behandeling van ovariumkanker verminderen. *In vitro* studies laten zien dat het combineren van conventionele chemotherapie met DR-gerichte medicijnen synergistisch kan werken, met een toename van celdood in zowel chemogevoelige als -resistente ovariumkankercellijnen.[18,19] Het bestuderen van de mechanismen die ten grondslag liggen aan de versterkte gevoeligheid voor rhTRAIL

door conventionele chemotherapie kan het best worden bestudeerd in isogene cellijnmodellen. In **Hoofdstuk 5** beschrijven wij onderzoek naar de moleculaire determinanten die bepalend zijn voor gevoeligheid voor rhTRAIL in een isogeen ovariumkankercellijnmodel met differentiële gevoeligheid voor platine-bevattende chemotherapie. In dit model hebben wij tevens gekeken naar de mechanismen die leiden tot de synergie tussen rhTRAIL en cisplatine. Platine gevoelige (A2780) en resistente (CP70, een dochtercellijn van A2780) ovariumkankercellen werden blootgesteld aan rhTRAIL en/of cisplatine. A2780 cellen bleken gematigd gevoelig voor apoptose inductie met rhTRAIL, terwijl CP70 cellen hiervoor resistent bleken. De combinatie rhTRAIL *plus* cisplatine resulteerde in sterk toegenomen apoptose-inductie in beide cellijnen. Dit kon worden verklaard door toegenomen expressie van caspase-8 eiwit en mRNA na blootstelling aan cisplatine, met name in CP70 cellen. Onbehandelde CP70 cellen hebben een lagere expressie van caspase-8 eiwit dan de moedercellijn A2780, terwijl de mRNA expressie tussen beiden niet verschillend bleek. Afbraak van caspase-8 mRNA en eiwitstabiliteit van caspase-8 waren gelijk in beide cellijnen, ongeacht de aan- of afwezigheid van cisplatine. Door de expressie van caspase-8 te verminderen (met 'small-interfering' RNA, siRNA) of te verhogen (met overexpressie constructen), konden wij aantonen dat de inductie van caspase-8 eiwit door cisplatine essentieel is bij het gevoelig maken van ovariumkankercellen voor rhTRAIL. Additionele experimenten met c-FLIP en p53 siRNA lieten zien dat noch een veranderde caspase-8/c-FLIP ratio noch een p53-afhankelijke toename in DR5 expressie op de celmembraan hierbij betrokken is. Uit deze experimenten concluderen wij dat cisplatine de potentie van rhTRAIL versterkt om apoptose te induceren in platine resistente en gevoelige ovariumkankercellen, waarbij inductie van caspase-8 eiwit expressie een sleutelrol speelt.

In **Hoofdstuk 6** werd gekeken naar de potentie van APO010 om apoptose te induceren in een panel van 12 kankercellijnen afkomstig van verschillende solide tumortypen met uiteenlopende resistentieprofielen, waaronder platine resistente en gevoelige ovariumkankercellen. Er werd gekeken naar de potentie van APO010 om chemoresistentie alsmede resistentie voor DR-gerichte medicijnen te omzeilen. De cellijnen binnen het panel waren gegroepeerd in drie isogene resistentiemodellen, waarbinnen werd gekeken naar celoverleving, inductie van apoptose, caspase-3 activatie, membraanexpressie van Fas en de expressie van DISC eiwitten. In meerdere solide kankercellijnen, waaronder A2780 en CP70 ovariumkankercellen, werd vermindering van celoverleving en toegenomen inductie van apoptose waargenomen

na blootstelling aan APO010. De gevoeligheid van cellijnen voor APO010 was onafhankelijk van intrinsieke chemoresistentie. Fas membraanexpressie correleerde kwalitatief met de gevoeligheid voor APO010, en was het hoogst in de ovariumkankercellijnen vergeleken met cellijnen afkomstig uit andere solide tumortypen. Voorbehandeling met cisplatine resulteerde in opregulatie van Fas en verhoogde de gevoeligheid voor APO010, onafhankelijk van intrinsieke platine resistentie of initiële ongevoeligheid voor APO010 monotherapie. Deze data laat zien dat APO010 leidt tot sterke inductie van apoptose in verscheidene solide kankercellijnen, waarbij geen sprake is van kruisresistentie tussen APO010 en conventionele chemotherapie. Dit impliceert een rol voor APO010 in het omzeilen van chemoresistentie. Tot slot leidt combinatie van APO010 met platine-bevattende chemotherapie tot een versterkt antitumor effect.

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DANKWOORD
(ACKNOWLEDGEMENTS)

DANKWOORD

Een belangrijk deel van de dag-tot-dag charme van onderzoek is voor mij gelegen in het samenwerken met een breed scala aan professionals en persoonlijkheden. Biologen, farmaceuten, medici allerhande, analisten, studenten vroeg in de opleiding, en post-docs of professoren met een sterkgewortelde visie, allemaal met een eigen invalshoek en expertise. Los van het gegeven dat deze smeltkroes mij een plezierige en uitdagende promotietijd heeft geboden, ben ik velen dank verschuldigd voor de totstandkoming van dit proefschrift. Een aantal personen wil ik hier graag nader noemen.

'First and foremost' gaat mijn dank uit naar mijn promotores Prof. Dr. E.G.E. (Liesbeth) de Vries, Prof. Dr. A.G.J. (Ate) van der Zee en Prof. Dr. S. (Steven) de Jong, en mijn copromotores Dr. A.K.L. (An) Reyners en Dr. H. (Hetty) Timmer-Bosscha.

Beste Liesbeth, het wordt voor jouw promovendi alsmaar lastiger een origineel dankwoord te produceren met de vele succesvol afgeronde en succesvol lopende onderzoeksprojecten die aan jouw naam verbonden zijn. Jouw visie, begrip, overzicht, enthousiasme, probleemoplossend vermogen en arbeidsethos zijn onovertroffen. Als het einde zoek was en ik op het punt stond een scheurkalender van de data te maken, wist jij er altijd weer in een handomdraai structuur en betekenis aan te geven. Niets ontglipt de aandacht wanneer jij de correctiestift hanteert, wat elk stuk inhoudelijk en tekstueel boven zichzelf uit heeft doen stijgen. De gelegenheid en wetenschappelijke vorming, maar zeker ook de genoten vrijheid die jij mij als promovendus hierbinnen hebt geboden, zijn voor mij een voorrecht geweest.

Beste Ate, jouw vermogen tot logisch en kritisch redeneren is bewonderenswaardig. Jij weet als geen ander de sterktes, maar zeker ook de zwaktes van een studie te onderkennen, opdat zij kunnen worden ondervangen. Hoewel jouw charisma menig promovendus weleens heeft belemmerd in de uitvoering van een vurig betoog, sta je altijd open voor verschillende zienswijzen en interpretaties. Met jou van gedachten wisselen is voor mij zowel een uitdaging als een plezier geweest.

Beste Steven, wij kennen elkaar al vanaf het moment dat ik als nitwit werd geïntroduceerd in de wereld van het laboratoriumonderzoek. Jouw legendarische uiteenzettingen op het whiteboard hebben mijn interesse in celbiologische

mechanismen aangewakkerd. Ook de passie voor (cellijn)experimenten heb ik voor een belangrijk deel aan jou te danken. Ik denk met genoegen terug aan de late uren die wij hebben besteed aan het nader analyseren van blots en curves. Jouw kennis, creativiteit, interesse en discussievaardigheid, altijd gepaard gaande met een goede dosis humor, maakt van jou een inspirerend begeleider en mens.

Beste An, als 24/7 begeleider ben jij van enorme waarde geweest. Jij was altijd op de hoogte van mijn activiteiten, en wist altijd het overzicht te behouden. Met subtiele hints wist je altijd haarfijn mijn prioriteiten te herschikken. Jouw inzicht in het natuurlijke beloop van een promotietraject heeft mij gerustgesteld. Jij hebt mij geleerd de uitkomsten van klinische studies te begrijpen en te interpreteren. Hoewel het mij meer dan eens een kop koffie heeft gekost, kon ik altijd bij jou terecht voor advies.

Beste Hetty, het woord 'copromotor' doet vermoeden dat de Nederlandse taal werkelijk een woord kent waarin jouw rol op het lab in één zucht is te noemen. Niets is minder waar. Met jouw praktische insteek en doortastende aanpak was elk ontaardend experiment in goede banen te leiden. Bij jou kon ik altijd binnen komen vallen. Binnen de kortste keren weet jij een reddingsplan op te stellen, meestal voor het experiment, soms voor de promovendus. Voor elk probleem had jij een oplossing of advies, daar heb ik veel aan gehad.

Mijn dank gaat tevens uit naar de verscheidene co-auteurs die meewerkten aan de verschillende hoofdstukken van dit proefschrift. Enkelen van hen wil ik in het bijzonder noemen. Beste Derk Jan (Dr. D.J.A. de Groot), de tijd die jij hebt geïnvesteerd om mij 'tips and tricks' bij te brengen in het lab hebben niet alleen de basis gelegd voor een hoofdstuk, maar voor dit hele proefschrift. Jouw aanpak heb ik altijd als voorbeeld gezien. Beste Evelien (Dr. E.W. Duiker), ook jou bedank ik voor de kans die ik kreeg mijn onderzoeksinteresse verder vorm te geven. Anton (Terwisscha-van Scheltinga), aan onze schrijfsessies die werden ingeleid door een nummertje 34 met nasi zal ik altijd goede herinneringen houden. Prof. Dr. H. Hollema en Dr. W.F.A. den Dunnen, beste Harry en Wilfred, jullie hebben mij de grote schoonheid van kleine stukjes weefsel doen inzien. Beste Annemieke (Dr. A. Meijer), als Western blotten ooit een Olympische sport wordt, of TEMED officieel als biologisch wapen wordt erkend, ben je de eerste die ik bel. Bedankt voor alle leuke tijden in het eiwitlab.

Graag wil ik alle analisten werkzaam op het Multidisciplinair Oncologisch Laboratorium bedanken voor de geboden hulp. Enkelen van hen wil ik specifiek noemen. Beste Phuong (Le) en Wytske (Boersma-van Ek), of het nu ging om MTT assays, kleuringen of celkweek, jullie wisten altijd de tijd te vinden om er een experiment bij in te passen. Ik ben jullie dankbaar voor de hulp. Beste Klaske (ten Hoor), het enthousiasme waarmee jij keer op keer (PhD-)studenten wist te charmeren voor de immunohistochemie kenmerkt jou. Samenwerken met jou was altijd een plezier. Beste Gert Jan (Meersma), de precisie en voorbereiding waarmee jij je experimenten uitvoert is voor iedereen een voorbeeld. Beste Linda (Pot), voor de dierstudie heb jij belangrijk werk verricht. Jij combineert moeiteloos een moderne ELISA kit met traditioneel keukengerei als een appelboor en een mixer.

Beste Dr. J. Meijer, beste Coby, jouw mentoronderwijs was de basis voor mijn interesse in oncologisch onderzoek. Samen met Hetty beman jij de commandopost op het lab. Jouw financiële vernuftigheid heeft ons vele gratis 'test'-kitjes opgeleverd, waarmee de experimentendrang kon worden bevredigd.

Beste Gretha (Beuker) en Bianca (Smit-Aeilkema), bedankt voor alle secretariële ondersteuning tijdens het submitten van artikelen en het insturen van dit proefschrift op vrijdag de 13e.

De leden van de leescommissie, Prof. Dr. F.A.E. Kruyt, Prof. Dr. A.J. Moshage en Prof. Dr. H.W. Nijman, wil ik danken voor de beoordeling van dit proefschrift.

Ook wil ik de programmagroep van de Junior Scientific Masterclass (JSM) bedanken, die mij de gelegenheid heeft geboden het MD/PhD-traject te volgen. Jullie doelstelling en inzet maakt het voor vele jonge onderzoekers mogelijk hun vleugels uit te slaan.

Beste collegae en oud-collegae van alle disciplines werkzaam binnen het Multidisciplinair Oncologisch Laboratorium, ik heb enorm genoten van de tijden die wij samen in en buiten het lab hebben doorgebracht. Op geen andere werkvloer vind je zo'n gemêleerd gezelschap. De discussies die ik met jullie heb genoten, de (veelal slechte) grappen en de lange gesprekken tijdens onze urenlange sessies in de celkweekruimte hielden elk monotoon werk draaglijk. Beste medestanders in de feestcommissie, onze labdag kan simpelweg niet worden overtroffen. Wie het heeft

geregeld weten we nog steeds niet, maar het budget leek maar geen grenzen te hebben. Ook wil ik de diverse kamergenoten die ik heb genoten bedanken voor hun begrip voor het concept van de 'kamerhit'. Graag zou ik jullie allemaal individueel in het zonnetje zetten, maar daar is simpelweg niet genoeg oerwoud voor over. Dear Malgosia (Krajewska), Karin (Tamas) and Bodvaël (Pennarun), for you I'd like to make an exception and add some English courtesy to keep you from judging the Dutch. I thank you for expanding my horizon and understanding of different cultures, and the opportunity to tweak my English. It has been a privilege.

Beste familie en vrienden, dispuutgenoten, (oud-)huisgenoten en medecoassistenten, zonder jullie begrip en steun was dit proefschrift niet tot stand gekomen. Velen van jullie hebben mij wel eens zien worstelen op dagen dat ik het licht even niet meer zag, en wisten mijn zorgen moeiteloos te relativeren met een goed gesprek, een luisterend oor, een corrigerende tik of, veelal, een excursie door de plaatselijke horeca. Jullie herinneren mij eraan waar Noord en Zuid liggen, als mijn eigen kompas het af laat weten.

Beste pap en mam, het meest relevante experiment hebben jullie 26 jaar geleden ingezet. Ik ben jullie meer verschuldigd dan wie dan ook. Jullie hebben mij altijd de vrijheid en kans geboden mijn eigen keuzes te maken, mijn eigen fouten te maken en mijn eigen lessen te trekken. De waarde van enkele onwrikbare overtuigingen en idealen, en de maakbaarheid van het eigen leven heb ik van jullie geleerd. Zonder jullie was ik een minder mens geweest.

Beste Emma, lieve zus, in sommige zaken elkaars tegenpolen, in sommige zaken 2 handen op 1 buik. Ik bewonder jouw standvastigheid en levenslust, en de scherpe tongval waarmee je mij bij vlagen (terecht) op mijn plaats zet. Ik had geen leukere zus kunnen wensen.

Tot slot wil ik mijn paranimfen in het bijzonder danken: Willem van der Bilt, Kirill Pavlov en Marten Nijhuis.

Beste Willem, beste broer, jij en ik hebben veel veranderingen samen doorgemaakt. Ik kan genieten van jouw brede interesse, jouw nieuwsgierigheid, jouw intelligentie, jouw

intrinsieke humor en onvoorspelbaarheid. Met jou zul je niet snel een saai moment beleven.

Beste Kirill, het laatste jaar van mijn onderzoek ben jij erg belangrijk geweest voor zowel dit proefschrift als mijn gezondheidstoestand. Jij wist als geen ander in tijden van stress mij te ontlasten. In Nepal betaal ik jouw ontbijt. Onze momenten als lab DJ's zullen niet snel worden vergeten. Wij delen vele interesses, zowel op eigen bodem als daarbuiten.

Beste Marten, wij kennen elkaar inmiddels alweer 8 jaar en toch levert elke keer dat wij elkaar treffen weer een originele herinnering op. Om jouw sociale natuur en spontane karakter kan niemand heen, en jij geeft meedenken een andere dimensie. Ik hoop dat wij onze gedeelde levenslust tot in het bejaardenhuis weten vast te houden.

ARNE